Development of a microfluidic device for single cell analysis using FT-IR microscopy

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Abstract

Submitted by Francis John Ball
For the Degree of Doctor of Philosophy and entitled:
Development of a microfluidic device for single cell analysis using FT-IR microscopy

Prostate cancer is the second most common cause of cancer fatalities in males in the UK (2006) [1]. Therefore any advances in the diagnosis or screening for this form of cancer will yield significant benefits in the treatment of this disease. FT-IR has already been successfully used to assess and grade prostate biopsies by Gazi et al 2006 [2]. The collection of prostate biopsy is however a highly invasive procedure and as current screening methods are highly sensitive, but not very specific, large numbers of patients are referred for biopsy procedures that later come back as negative for prostate cancer [3]. Harvey et al used Raman spectroscopy to classify live cells of a number of prostate cell lines as a first step towards a possible urine screening protocol for prostate cancer [3]. Due to the complementary nature of Raman and FT-IR spectroscopy a similar live cell study should be possible using FT-IR and the combination of this technique with a high-throughput microfluidic device could lead to a useful screening tool for prostate cancer.

The aim of the project was therefore to develop a microfluidic system which would enable higher through-put FT-IR analysis of live single cells in an aqueous carrier solution such as PBS or urine than has been previously possible. The design of the microfluidic device must also account for the fact that the materials used to produce the analysis chamber must be highly transparent to mid-IR radiation. The microfluidic device and peripheral systems must be easily transportable as it will be necessary to perform experiments in multiple locations. A design and manufacturing protocol for such a device has been developed.

The development of a spectral contribution removal algorithm for the aqueous carrier fluid will also be necessary in order to allow the accurate interpretation of the IR data obtained. A least squares fitting based spectral subtraction algorithm was developed and validated for this purpose.

Although it did not prove possible during the project to investigate the possible application of this device to a prostate cancer screening protocol other applications in cell line classification and drug cell interaction studies were performed and yielded encouraging results.
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<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>Aβ</td>
<td>β-amyloid</td>
</tr>
<tr>
<td>ATRA</td>
<td>Trans-retinoic acid</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic force microscopy</td>
</tr>
<tr>
<td>APP</td>
<td>Amyloid precursor protein</td>
</tr>
<tr>
<td>AUA</td>
<td>American urological society</td>
</tr>
<tr>
<td>BHP</td>
<td>benign prostatic hyperplasia</td>
</tr>
<tr>
<td>cm(^{-1}) or WN</td>
<td>Wavenumber</td>
</tr>
<tr>
<td>Co-scans</td>
<td>Co added scans</td>
</tr>
<tr>
<td>DF</td>
<td>discriminant function</td>
</tr>
<tr>
<td>DSMZ</td>
<td>Deutsche Sammlung von Mikroorganismen und Zellkulturen</td>
</tr>
<tr>
<td>DOX</td>
<td>Doxorubicin</td>
</tr>
<tr>
<td>DRE</td>
<td>Digital rectal examination</td>
</tr>
<tr>
<td>ECACC</td>
<td>European Collection of Cell Cultures</td>
</tr>
<tr>
<td>ECM</td>
<td>Extra cellular matrix</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FPA</td>
<td>Focal Plane Array</td>
</tr>
<tr>
<td>FT-IR</td>
<td>Fourier transform infra red</td>
</tr>
<tr>
<td>Ge</td>
<td>Germanium</td>
</tr>
<tr>
<td>GeV</td>
<td>giga electron volt</td>
</tr>
<tr>
<td>Gln</td>
<td>L-Glutamine</td>
</tr>
<tr>
<td>H(_2)O</td>
<td>Water</td>
</tr>
<tr>
<td>Hr(s)</td>
<td>Hour(s)</td>
</tr>
<tr>
<td>Hz</td>
<td>Hertz</td>
</tr>
<tr>
<td>IR</td>
<td>Infra red</td>
</tr>
<tr>
<td>IRENI</td>
<td>Infrared environmental imaging</td>
</tr>
<tr>
<td>KBr</td>
<td>Potassium bromide</td>
</tr>
<tr>
<td>keV</td>
<td>kilo electron volt</td>
</tr>
<tr>
<td>LDA</td>
<td>Linear discriminate function analysis</td>
</tr>
<tr>
<td>Linac</td>
<td>Linear accelerator</td>
</tr>
<tr>
<td>MALDI-TOF MS</td>
<td>matrix assisted laser desorption ionisation time of flight mass spectrometry</td>
</tr>
<tr>
<td>MeV</td>
<td>mega electron volt</td>
</tr>
<tr>
<td>mW/cm(^2)</td>
<td>milliwatts per square centimetre</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Min</td>
<td>Minute(s)</td>
</tr>
<tr>
<td>NaNs</td>
<td>Not-a-number</td>
</tr>
<tr>
<td>ng/ml</td>
<td>nanograms per millilitre</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline solution</td>
</tr>
<tr>
<td>PC</td>
<td>Principal component</td>
</tr>
<tr>
<td>PCA</td>
<td>Principal component analysis</td>
</tr>
<tr>
<td>Pen\Strep</td>
<td>Penicillin and Streptomycin</td>
</tr>
<tr>
<td>PMMA</td>
<td>Poly (methyl methacrylate)</td>
</tr>
<tr>
<td>PMT</td>
<td>Photomultiplier tube</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate serum antigen</td>
</tr>
<tr>
<td>RF</td>
<td>Radio frequency</td>
</tr>
<tr>
<td>RMieS</td>
<td>Resonant Mie scattering correction</td>
</tr>
<tr>
<td>RPM</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>s</td>
<td>Second(s)</td>
</tr>
<tr>
<td>SNR</td>
<td>Signal to noise ratio</td>
</tr>
<tr>
<td>SR</td>
<td>Synchrotron radiation</td>
</tr>
<tr>
<td>tet</td>
<td>tetracycline</td>
</tr>
<tr>
<td>TRUS</td>
<td>Trans rectal ultra sound</td>
</tr>
<tr>
<td>VACURG</td>
<td>Veterans Administration Cooperative Urological Research Group</td>
</tr>
<tr>
<td>µm</td>
<td>micrometers</td>
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</tbody>
</table>
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Thank you all, I would not have got this far without all your help and support.
CHAPTER 1  Introduction

1.1  The biology of cancer

Cancer is an umbrella term for over 200 different diseases which arise as a result of uncontrolled cellular proliferation [1, 4]. These various forms of cancer are broadly classified according to the cell type in which the primary tumour originates. The most common class of cancer are the carcinomas. These occur in epithelial cells found in organs such as the skin and lungs. The next class of cancer is the sarcomas which occur in the mesenchyme (supporting tissue). Some examples of the tissues affected by this class of disease are the smooth muscle, bone and cartilage. The third class of tumours are the lymphomas. These arise in the cells of the immune system and lymphatic tissues. Leukaemia is another class of cancers which occur among the immature blood cells in the bone marrow and then accumulate in the blood stream. The class of cancer is identified by the use of the appropriate prefix. This denotes the exact cell type in which the cancer originated; for example Adeno would indicate a glandular cell type whereas Osteo would indicate bone as the tissue of origin [5-6].

The reason all these different diseases are referred to as a single condition is because they all arise in a similar fashion. That is by cells acquiring a series of genetic mutations. These changes to the cellular DNA and therefore the gene expression profile of the cell result in a change in the cells response to stimuli. This allows the cell to gain a number of advantages over other normal cells at which point the cell beings to exhibit parasitic instead of the usual symbiotic behaviour of its neighbours [6]. The advantages gained include the reacquisition of cell immortality and the ability to proliferate at a higher rate than normal by reducing the response to growth suppression cell signalling pathways. One of the most important differences however, between normal and cancerous cells is the ability of cancer cells to resist apoptotic signalling. This becomes particularly problematic for the affected individual when the anoikis pathway is suppressed. Anoikis is the process which triggers apoptosis in cells when they become separated from their native extra cellular matrix (ECM) and cellular neighbours. Therefore the suppression of anoikis allows the free movement of the cancer cells throughout the body. This is the point at which the cancer is termed metastatic [4, 7].
1.2 Prostate Cancer

Prostate cancer is the most common cancer in men and accounts for around 25% of all new cancer diagnoses [1]. The prostate is the largest accessory gland in the male reproductive system. The tissue composition of the prostate is around 2/3 glandular tissue with the final 1/3 being smooth muscle tissue [8]. The prostate gland is located below the bladder and behind the penis; the urethra passes through the centre of the gland as depicted in Figure 1:1 [9].

![Prostate gland diagram](image)

**Figure 1:1** The prostate gland is situated at the base of the bladder behind the penis. The urethra passes through the centre of the gland. Figure is adapted from [9].

The prostate is responsible for the production and secretion of certain components of the seminal fluid such as prostate specific antigen (PSA) into the urethra. The vast majority (over 90%) of prostate tumours are adenocarcinomas occurring in the luminal epithelial tissue of the prostate. The luminal epithelial tissue is the outer most layer of cells which line the ducts within the prostate [5].

1.2.1 Screening for prostate cancer

There are currently two used screening techniques which are considered to be the clinical standard by the American urological association (AUA) for diagnosing prostate cancer these are the digital rectal examine (DRE) and the PSA screening methods. These techniques are usually employed in combination as they complement each other very well [10].
1.2.1.1 Digital Rectal Examination (DRE)

Until recently the DRE was the most commonly employed initial test for the diagnosis of prostate cancer. The examination is performed by palpation of the prostate through the wall of the rectum. This is possible due to the close proximity of the prostate gland to the rectal wall. The examination is able to determine the size, symmetry and texture of the prostate. The presence of any lesions can also be detected from this technique [10-11].

The advantages of this method of examination are that it is simple and inexpensive. Unfortunately there are also some limitations. Around 40% of prostate cancers which occur in the anterior to midline of the prostate cannot be detected as this region cannot be palpated during DRE. It is also very difficult to detect small lesions particularly those of which are < 1.5 cm in size [10, 12].

A recent study by Bosch *et al.* found that DRE was of limited value in determining prostate volume when the prostate is less than or greater than 50 ml [13]. This is because DRE tends to overestimate prostate volume in smaller prostates and underestimates prostate volume in larger ones [13]. There are however some limitations in this study which were discussed in a recent review by Kijvikai [14]. The review states that the study only achieved a 50% response rate with the non-respondents going unevaluated [14].

1.2.1.2 Prostate-Specific Antigen (PSA) Test

PSA is a serine protease produced by prostatic epithelial cells. The main function of PSA is to liquefy semen during ejaculation [15]. PSA is concentrated within prostatic tissues however a small amount is normally present in blood serum [10]. The most commonly used assay for determining PSA serum levels is the Tandem®-R assay. There are three classifications for the results obtained from the assay which are normal, borderline and high. Patients in the normal class will have PSA serum levels of 0-4 nanograms/millilitre (ng/ml). The PSA level is said to be high if it is greater than 10 ng/ml, and borderline between 4 and 10 ng/ml [10-11].

The major problem with using PSA serum levels as a diagnostic test is that they are related to the size of the prostate and not directly to the presence of a malignant cancerous tumour. There are for example a number of conditions which can affect the size of the prostate and therefore induce a rise in PSA levels but are not
malignant cancers. A couple of examples of this are prostatitis, which is an infection of the prostate that causes the prostate to swell and benign prostatic hyperplasia (BPH), a condition that results in the over growth of prostate epithelial and stromal cells which produce nodules on the prostate. These growths however are completely benign and non-invasive. These conditions however, are not the only cause of raised PSA levels. Indeed ejaculation as well as mechanical manipulation of the prostate may also cause PSA levels to rise. It is therefore possible for procedures such as biopsy and catheterisation to elevate PSA levels as they can result in this form of prostate manipulation [10-12, 15].

In order to increase the sensitivity and specificity of PSA in prostate cancer detection, four techniques are used. These are age-specific PSA which takes into account the fact that the prostate increases in size as men age as well as the ethnic background of the patient; PSA density which is the PSA serum level divided by the transrectal ultrasonography (TRUS) determined prostate volume; PSA velocity which uses the increase in PSA serum levels over time to determine whether a cancer is present and finally, free-to-total PSA which uses the ratio of bound and unbound PSA in the blood to determine the presence of prostate cancer with a lower ratio indicating the presence a malignant prostate cancer [10-11, 16].

1.2.1.3 Transrectal Ultrasonography (TRUS)

TRUS is the industry gold standard technique for the determination of prostate volume. This technique uses ultrasound to visualise the prostate and allow calculation of prostate volume [14]. This technique however is not suitable for primary screening due to the high costs. This technique also has difficulty in detecting lesions in the transitional zone of the prostate, as well as low predictive values in small lesions [10].

1.2.2 Diagnosis of prostate cancer

While the previously discussed methods are very useful for screening in order to indicate the possible presence of cancerous tumours, the only universally recognised method for diagnosis of prostate cancer is to obtain a biopsy. The biopsy must then be viewed by a trained histopathologist who evaluates and grades it [17]. The biopsies are obtained under TRUS guidance using a spring-loaded
biopsy gun fitted with a cored needle [10-11]. This produces a needle core biopsy, the process is usually repeated multiple times from different areas of the prostate in order to obtain a more accurate assessment of the prostate's condition [10].

1.2.2.1 Gleason grading system

The Gleason grading system is a histological grading system which was developed from the combined data generated in the original Veterans Administration Cooperative Urological Research Group (VACURG) studies conducted between 1960 and 1975 as well as other VACURG follow up studies [18]. The grading system is based on nine histological patterns which are identified by their unique appearance. These patterns were then grouped based on survival data. This revealed that certain patterns often occurred together and shared similar mortality rates. The patterns were then grouped into 5 distinct grades consisting of 3 sets of related patterns and 2 distinct patterns. The grades were then ordered by combining malignancy and mortality data and finally graded accordingly from 1 to 5 with 5 being the most aggressive cancer [18].

Unfortunately around half of the tumours graded using this system appeared to contain regions of different histological grades. This led to the development of the scoring system which is now referred to as a Gleason score. This score is generated by adding both the Gleason grades of the tumour together but not dividing the product by 2. The Gleason score is therefore a number between 2 and 10. The additional integers are necessary because prostate tumours with multiple Gleason grades do not have a mortality rate equal to the highest grade but rather appear as an average of the two grades and so the Gleason score was developed as a method of predicting this tumour behaviour. The appearance of prostate tissue at varying Gleason grades is shown in Figure 1:2 [18].
Figure 1:2 The figure depicts the cellular architecture at the different Gleason grades. A grade 1 gland appears as simple rounded masses with well-defined edges. As the Gleason grade increases the cellular architecture degenerates until grade 5 at which point the glands have lost all differentiation. Cells are present as solid sheets, cords, solid tumours with central necrosis or as single cells. Figure adapted from [18].

Although histological grading is widely accepted as the gold standard for determining the prognosis and therefore treatment of a patient, it is also widely accepted that this kind of evaluation is highly subjective and so leads to interobserver and intraobserver variation [18-20]. In order to remove these kinds of variation it is necessary to assess the biochemistry of the cells within the tissue and not just the tissue architecture, one method of doing this is by Fourier transform-Infrared (FT-IR) microspectroscopy as shown by Gazi et al. in 2006 [2].

1.2.3 Vibrational spectroscopy in prostate cancer

The two vibrational spectroscopy techniques commonly used for prostate cancer research are FT-IR and Raman spectroscopy.
1.2.4 Raman spectroscopy in prostate cancer

Some of the early work involving Raman spectroscopy and prostate cancer was performed by Crow et al. [21-23]. This work demonstrated how Raman spectroscopy could be used to differentiate between prostate cancer cell lines and in grading prostate cancer [21-22]. This work was further developed by Harvey et al. who used live cell Raman spectroscopy to discriminate between different urological cell lines with the eventual aim to develop a screening procedure for prostate cancer based on a urine sample [3].

1.2.4.1 FT-IR microspectroscopy in Prostate cancer

Some of the early work on the possible use of FT-IR microspectroscopy in the diagnostic process of prostate cancer was undertaken by Gazi and co-workers [24]. Gazi et al. were able to demonstrate that FT-IR microspectroscopy could distinguish between benign and malignant epithelial cells within tissue samples, as well as malignant cells derived from other metastatic sites. The data also indicated a chemical difference between BPH and normal prostate tissue [24]. This was later confirmed and refined with the use of more rigorous data analysis techniques into a protocol able to classify tissue samples with a sensitivity of 83.6% and specificity of 86.0%, far higher than the currently used histopathology [17]. However, while the use of FITR tissue analysis may in the future help to improve the diagnosis of prostate cancer from the obtained biopsies, this is not the only possible role for FT-IR in the diagnosis of prostate cancer.

One of the largest problems with the current screening and diagnostic process for prostate cancer is that the screening procedures used, while very sensitive, are not very specific. This means that large numbers of patients are referred for biopsy procedures that later come back as negative for prostate cancer [3]. For example a positive PSA test result corresponds to a positive cancer diagnosis in only 22% of cases [3]. Indeed vibrational spectroscopy in the form of Raman spectroscopy has already been used to investigate PSA levels and their relationship with prostate cancer [25-26].
1.3 Single cell FT-IR analysis

The work of Gazi et al. was not however limited to purely tissue samples. Indeed work performed on a number of prostate cancer cell lines proved FT-IR spectra could be used to clearly differentiate between closely related prostate cancer cells lines [24]. The same group also used single cell FT-IR spectroscopy to study CaP cell motility and cytokinesis [27]. Although they were not the first to perform such a study of cytokinesis, that study was conducted by Jamin et al. in 1998. Jamin and co-workers used Synchrotron Radiation (SR) FT-IR to generate chemical maps of single cells, and found a difference in the distribution of proteins and lipids, in the final stages of cell division and necrosis [28]. The use of single cell FT-IR has also been used by several groups to study a wide variety of topics such as the identification of rare cell phenotypes [29], cell cycle, cell death [30], cell fixation protocols [31], cell scattering artefacts [32-34] and even drug-cell interactions [35-37].
There are two main methods of collecting single cell FT-IR spectra, using a synchrotron or a Focal Plane Array detector (FPA). It is also possible however, to obtain single cell spectra using a standard benchtop FTIR spectrometer and detector. However, the signal to noise ratio of the cell spectrum obtained is not as good as that of a cell spectrum obtained using either a synchrotron source or an FPA detector. The collection of a single cell spectrum using a synchrotron source involves closing the aperture to a size similar to that of a single cell, around 10-15 micrometers (μm). In order to gain a suitable single to noise ratio (SNR) at these small aperture sizes a different kind of IR source is required, namely synchrotron radiation (SR) [38-39]. The SNR advantage of using SR over a thermal source at these small apertures is illustrated in Figure 1:3 [40]. This is discussed further in 2.1.3.1. The second method employs a standard IR thermal source but a different type of detector and is discussed further in 2.1.4.

Figure 1:3 comparison of signal to noise ratio using a thermal source and a synchrotron source at various aperture sizes. The synchrotron data is represented in red whereas data obtained from a thermal source is shown in blue [41].

1.3.1 Live cell FT-IR

As spectral interference from water does not interfere as much with the Raman signal, this method has been used previously for live cell analysis. However, there are a number of problems with Raman spectral analysis [3, 42]. The first of these is that Raman spectroscopy is prone to poor SNR which leads to the requirement for longer data acquisition times. A second common issue with Raman spectroscopy,
particularly with biological samples is autoflorescence of samples, which can dominate the spectra acquired. The last common issue with Raman spectroscopic analysis of biological samples is that the use of the high powered laser source required for this type of analysis can result in photo-damage to the sample under investigation [43-44]. FT-IR analysis however does not suffer from these particular disadvantages and as such the development of a device capable of obtaining live cell FT-IR spectra is a worthy endeavour.

The use of live as opposed to fixed cells for FT-IR experiments is an emerging field of interest. This is an area of increasing interest because the use of live cells as opposed to fixed samples confers a number of advantages, namely the ability to study cell responses to stimuli in real time [45-46]. The use of live cells is also advantageous because fixation process can affect the spectra obtained from biological samples [46]. It is also preferable to analyse hydrated biological samples because dehydration can affect the conformation of biomolecules such as proteins and nucleic acids [47-48]. Although earlier work was carried out by several groups, the devices used had pathlengths of 10-12 μm which made accurate interpretation of the amide I band difficult [46, 49-50]. The earliest devices sandwiched a thin film spacer between IR substrates, these films were composed of materials such as Teflon [46, 49-50]. These early devices could be classified in to two categories: static cells and flow cells.

1.3.1.1 Static cells

This class is the simplest form of device for live cell analysis. The earliest of which were like those used by Moore et al. and Miljkovic et al. [49-50]. This type of device has been greatly improved by the recent application of microfabrication techniques such as the use of photoresist layers as spacers and wet etching pioneered by groups such as Tobin et al. and Birarda et al. [51-52]. The use of microfabricated spacers improves the accuracy of the pathlength defined within the device as well as offering improved methods of sealing the device [45]. The main limitation of this type of device is that it is not possible to replace the sample placed within them without dismantling the device. This reduces the applicability of these devices to more complicated live cell studies in two ways. The first is that it makes the device unsuitable for high throughput applications as it is not possible for the sample to be easily removed and replaced while keeping the device intact. The second limitation
of this type of device is, because it is not possible to change the growth medium the cells are maintained in, it is not possible to perform long *in situ* cell experiments with this type of device [51].

### 1.3.1.2 Flow cells

This is a more complicated type of device usually designed for long time period experiments. The basic design consists of an inlet where cell growth medium can be introduced to the cells and an outlet were the growth medium exits the device after the cells have converted the nutrients to waste. Some examples of this type of device were presented recently by Marcsisin *et al.* in 2010 [53]. This type of device can also be used for more complex studies such as the effect of changes in the physiological environment of the cells such a study that was performed by Birarada *et al.* [54]. It should be noted however that this more complex experiment requires a more complex device such as the device detailed in the same paper [54]. This type of device has not currently been used for a high throughput study, though the techniques used for the development of these devices could be applied to the development of such a device.

### 1.3.2 The water problem

One major difficulty of live cell analysis using FT-IR is the fact that water is a strong absorber of mid IR radiation. The FT-IR spectrum of water consists of three visible bands, the strongest of which is a broad absorption band resulting from the overlap of the OH symmetric and asymmetric stretching vibrations which occur between ~3500-3600 cm\(^{-1}\) and ~3700-3900 cm\(^{-1}\) respectively [55-56]. For the purposes of this thesis these two vibrations will in future be referred to as the OH stretching region. The second strongest visible band is the HOH bending mode at ~1640 cm\(^{-1}\) and the last is a combination band at ~2130 cm\(^{-1}\) (Figure 1:4) [54, 57]. The combination band results from the combined excitation of the HOH bending mode and librational bands which occur in the far IR between 500 and 800 cm\(^{-1}\) [54]. The OH stretching region is clearly saturated in Figure 1:4 which is a common phenomenon as a water layer of just 1 μm will produce this effect [54]. Detector saturation occurs when either too much or, as in this case too little IR radiation reaches the detector. This causes a non-linear detector response resulting in an
exaggerated peak height and in extreme cases a distortion of the spectra band [58]. However, as this region is not information rich in terms of cell spectra it can be discounted from the spectral analysis. Figure 1:4 also shows that the HOH bending mode has a significant absorbance. This can lead to problems when it comes to the interpretation of aqueous cell spectra because of the proximity of this water band to the amide I band of proteins. The close proximity of these two bands means that this region of the aqueous cell spectrum can easily become saturated, preventing the accurate interpretation of changes in the amide I band. As the amide I band is a major source of information on changes in protein chemistry in the cells under study, the exclusion of this band from the analysis could lead to important changes in protein chemistry being missed.

Figure 1:4 FT-IR spectrum of water. The OH stretching vibrations between ~3400 and ~3600 cm⁻¹ are the strongest absorbance band and can be seen to cause saturation of the detector. The second important absorbance band is the H-O-H bending mode at ~1640 cm⁻¹ [54, 57].

Water is however essential to live cell analysis as cells require an aqueous medium in order to survive. The key to producing high quality IR spectra of live cells is therefore to limit the bulk water layer to <10 μm to prevent saturation of the detector, but still maintain a thick enough layer for the cells to remain alive in [59].
1.3.3 Applications of live cell FT-IR

Although one possible use of the device designed for this project would be to further the work of Harvey et al. towards a screening methodology for prostate cancer there are many other possible applications for this technology [3, 60].

The most obvious alternative application of this technology is to perform a similar cytology based screening system for leukaemia as these cells are both generally small in size and non-adherent in phenotype making them ideal candidates for flow system analysis [61-62]. A study by Babrah et al. in 2009 has already shown how several leukaemia and lymphoma cell lines can be distinguished by a combination of FT-IR and multivariate statistical analysis [63]. However this study was limited to the use of air dried cells and the study was performed prior to the development of the Resonant Mie Scattering correction algorithm (RMieS) and therefore the data may have been subject to scattering differences. Another interesting type of study which could be investigated with this type of device are drug-cell interaction studies. One such study was performed using air dried samples by Mohammad et al., which centred on the treatment of lymphocytic leukaemia cells with bryostatin and detected a decrease in protein and DNA content accompanied by an increase in the lipid membrane [64]. Ramesh et al. used FT-IR to evaluate the progression of chemotherapy in childhood leukaemia although they were unable to correlate the spectral markers to the progression of the chemotherapy. The sample preparation method used in this study was again air drying of the samples [65]. The air drying sample preparation methodology is not an ideal sample preparation method, especially for drug cell studies as the air drying process can cause delocalisation of biomolecules due to the collapse of the internal cellular structure [31]. The removal of the cells from an aqueous environment and subsequent air drying can also lead to cell shrinkage or even cell enlargement and rupturing of the plasma membrane followed by the leakage of the intracellular components [31].

Another possible use for this device is in the field of stem cell research furthering the work of previous groups which have established that FT-IR can be used for this type of analysis [66-68]. However, these studies have been performed on fixed cells and as such, the data was acquired as average spectra which could result in rare phenotypic changes being missed as well as the spectra possibly suffering from scattering artefacts. It was not in fact until a recent study by Clemens et al. in to the
effect of retinoids on the differentiation of TERA.cl.SP12 embryonal carcinoma derived cell line that recent advances in scattering correction were applied to a stem cell differentiation study [69]. Although live stem cell spectra have been obtain previously by Zhao et al. they did not mention any spectral differences in the amide I region although the amide II was discussed [70]. This may have been due to the well-known problem of high water absorption in the amide I region preventing the analysis of this spectral band.

One other possible application of this device is as a means of validating the RMieS algorithm. In order to do this, a comparative study between spectral results obtained from an appropriate RMieS corrected formalin fixed cell study; with those obtained from an aqueous cell study should be performed. As the aqueous cell spectra should be free of Mie scattering if similar results were obtained from both studies this would provide good supporting evidence for the use of the RMieS algorithm. A suitable model cell system for this would be the MC65 cell line. The MC65 cell line is a stably transformed human neuroblastoma cell line that has been genetically modified to conditionally express a partial amyloid precursor protein (APP) fusion protein, which contains the full 99 amino acid residue carboxyl-terminus, but only 17 of the N-terminal amino acid residues. The conditional expression is controlled by the presence or absence of tetracycline (tet). In the absence of tet the APP fusion protein is expressed however, if tet is present the APP fusion protein expression is repressed [71]. APP is a transmembrane protein which after protein cleavage can produce β-amyloid (Aβ) [4]. Aβ is the polypeptide which aggregates into amyloid plaques which are found in the brain of patients diagnosed with Alzheimer’s disease (AD). AD is a neurodegenerative disease and is the most common form of dementia with around 496000 suffers in the UK alone [72]. As the brain is composed of highly organised tissue it is thought to be more vulnerable to the effects of protein aggregation in the ECM. These extracellular aggregates are often referred to as amyloid plaques. These are in turn formed from protein aggregates which have been released from dead neurons [4].
AD is a particularly useful model because the underlying biochemistry of plaque formation is well understood. The structure of the extracellular protein fibrils for example has been known for over 20 years since the work of Kirschner et. al. in 1985 [73]. In this form the Aβ peptides form both inter-molecular and intra-molecular β-sheet structures (Figure 1:5) [74]. In work by Jin et. al. in 2002 it was suggested that cell death was most likely triggered by intracellular accumulation of the C-terminal fragments of aβ peptides [71]. It wasn’t until 8 years later however that the secondary structures of these more neurotoxic oligomers were proposed by Ahmed (Figure 1:5) [74].

**Figure 1:5** models of amyloid aggregation as proposed by Ahmed et. al. the figure was adapted from [74]. a) depicts the monomeric form of aβ42 peptide present in the oligomeric conformation. The molecule is present in an anti-parallel β-sheet conformation. b) diagram of a pentameric oligomer. c) schematic of the monomeric aβ42 peptide present in fibrils. The molecule assumes an anti-parallel β-sheet conformation. d) proposed structure of a profilament. The aβ42 monomers pack together in parallel β-sheets.

The high level of β-sheet protein secondary structure in these oligomers, should lead to a detectable increase in the FT-IR spectral vibrations associated with this form of protein secondary structure. Therefore if a significant increase in these vibrations could be detected in both fixed and live cell FT-IR analysis of tet deprived MC65 cells then this would further validate the RMieS algorithm.
1.4 Aims

This project aims to develop a microfluidic system which will enable higher throughput FT-IR analysis of live single cells in an aqueous solution such as phosphate buffered saline solution (PBS) or urine than has been previously possible. The design of the microfluidic device must also account for the fact that the material used to produce the analysis chamber must be highly transparent to mid-IR radiation. The channel must be well sealed in order to prevent leakage of aqueous solutions. This is particularly important for two reasons, firstly that the samples under investigation may not reach the analysis chamber if the seal degrades and secondly leaks may damage the FT-IR instrumentation. This is due to the fact that many of the components of the instrument are vulnerable to damage by aqueous solutions as they contain mineral salt surfaces which can be degraded by contact with water. The microfluidic device and peripheral systems must be easily transportable as it will be necessary to perform experiments in multiple locations.

The development of spectral contribution removal algorithms for the aqueous carrier fluid will also be necessary in order to allow the accurate interpretation of the IR data obtained.

Another aim of this study is to validate the RMieS algorithm using the MC65 cell line.
CHAPTER 2   Experimental techniques

2.1 Infra Red spectroscopy

Infra red (IR) radiation covers the frequencies between $3 \times 10^{12}$ Hertz (Hz) and $3 \times 10^{14}$ Hz which corresponds to wavelengths between 1 μm and 100 μm [55]. The spectral band positions in IR spectra are reported using the historical convention of wavenumbers given the unit cm$^{-1}$ [55]. This is the inverse of the wavelength which provides a more convenient numbering system in interferometry [55]. The IR spectrum is divided into three regions: near, mid and far IR spectroscopy. For the purposes of this thesis only mid-IR spectroscopy has been used as strong absorbance bands for all the major biomolecule classes are present in this region between 400 and 4000 cm$^{-1}$. For practical reasons (e.g. cut-off of CaF$_2$ substrate) this has been further narrowed to between 3800 and 1000 cm$^{-1}$ (Figure 2:1). The absorbance band corresponding to the amide I located at ~1650 cm$^{-1}$ is particularly sensitive to changes in protein secondary structure which give rise to changes in the band shape.

![Figure 2:1 Example cell spectrum collected using FT-IR at 4 cm$^{-1}$ and 512 co-scans. The peaks corresponding to the major classes of biomolecules have been labelled.](image)

IR vibrational spectroscopy is based on IR light absorption by the sample; as such the absorbance bands in the spectrum correspond in frequency to the vibrational
modes of the covalent bonds between the atoms which make up the analyte molecule. An IR absorbance is present in frequency where the covalent bond absorbs a photon of the right energy for the molecule to enter the next vibrational energy level. This is determined by Equation 2-1.

**Equation 2-1**  \[ E = hv \]

Where \( E \) is the energy of the photon, \( h \) is Planck’s constant and \( v \) is the frequency [55]

The absorbance of a photon of energy equivalent to the difference in energy level between the ground and first vibrational excited state causes the bond to oscillate at the fundamental frequency for that molecular species. These oscillations can be molecular vibration like stretching or bending in solid state, as well as rotation and librations in gas and liquid phase.

Although IR analysis of diatomic molecules such as CO can have only a single fundamental absorption, polyatomic molecules produce many more. This is because polyatomic molecules are able to undergo \( 3N - 6 \) fundamental vibrations if it is non-linear and \( 3N - 5 \) fundamental vibrations if it is linear, where \( N \) is the number of atoms in the molecule [75]. This equation arises from the fact that every atom is able to occupy 3 different positions in 3 dimensional spaces independently of the other atoms within the molecule. These positions can also be referred to as ‘degrees of freedom’. The loss of 3 degrees of freedom stems from the fact that at any point in time the molecule may move as a whole around its centre of gravity and 3 degrees of freedom are required to account for this movement. The final 3 degrees of freedom are required to account for the rotation of the molecule as a whole around 3 perpendicular axes, however for a linear molecule there can be no rotation about the bond axis and therefore only 2 axes are required and so only 2 degrees of freedom are used [75].
In order to demonstrate this concept the IR activity of H\textsubscript{2}O will be discussed. H\textsubscript{2}O can be classed as a nonlinear, triatomic molecule. Therefore using the equation 3N – 6 it is possible to determine the number of fundamental vibration in this case 3. These fundamental vibrations are shown in Figure 2:2 however there is an important shift in the bending mode vibration from 1595 cm\textsuperscript{-1} to 1640 cm\textsuperscript{-1} when the H\textsubscript{2}O molecules change from the gaseous to liquid phase [54-55]. These vibrations are usually referred to as the ‘normal modes of vibration’ or ‘normal vibrations’ of the molecule [55].

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{water_vibrations.png}
\caption{A diagram of three fundamental vibrations of a water molecule. The vibrations represented are a) the symmetric bending, b) symmetric stretching and c) asymmetric stretching.}
\end{figure}

In order for a bond to be IR active, the vibrational state must result in a change in the dipole moment of the molecule. It should also be noted that the change in dipole moment is related to the intensity of the IR absorption band. This is illustrated in Equation 2-2 [75-76].

**Equation 2-2**

\[ I \alpha \left( \frac{\delta \mu}{\delta Q} \right)^2 \]

Where \( I \) is the intensity of the IR absorption band, \( \delta \mu \) is the change in dipole moment and \( \delta Q \) is the change in bond length [76].
2.1.1 Fourier transform Infrared (FT-IR) spectroscopy

FT-IR spectroscopy vastly improved the speed and sensitivity at which IR spectra could be collected. This decrease in the spectral acquisition time occurs because data is obtained from every wavenumber in the desired wavenumber range at the same time, as opposed to non FT-IR instruments which only collect a single wavenumber band at a time i.e. if data was being collected at 4 cm\(^{-1}\) resolution then a data point would have to be obtained every 4 cm\(^{-1}\) over the desired range sequentially, with a decrease in detected signal as higher wavenumber resolutions were employed [55].

The instrumental advancement which made this kind of analysis possible was the Michelson Interferometer. An example of one type of this kind of interferometer is pictured in Figure 2:3. This particular interferometer has a reference interferometer which is used to determine the exact position of the moving mirror, although other variations of the Michelson interferometer achieve the same result in other ways.

![Schematic diagram of a Michelson interferometer](image)

**Figure 2:3 Schematic diagram of a Michelson interferometer adapted from [77]**

The Michelson interferometer consists of two perpendicular mirrors (M1 and M2 Figure 2:3), one of which is fixed the, other moves between two set points. The
beam splitter bisects the two mirrors and must be made of a combination of materials capable of both transmitting and reflecting IR radiation; Germanium (Ge) coated Potassium bromide (KBr) is one commonly used substance with the Ge coating being just thick enough for 50% of the incident radiation to be reflected [58]. When IR radiation emitted from the IR source enters the interferometer, the beam is split into two parts by the beam splitter. One half of the beam is reflected to the fixed mirror (M1 Figure 2:3), the beam is then reflected straight back and is transmitted through the beam splitter. The beam then passes on to the detector. The second half of the beam is transmitted through the beam splitter initially, is then reflected back off the moving mirror (M2 Figure 2:3) and then passes back to the beam splitter where it is reflected back towards the detector. As M2 moves the path length, the two halves of the beam travel along will vary. The half of the beam passing through M1 will always have a path length of 2L where L is the distance between the M1 and the beam splitter (Figure 2:3) [77]. However, the second half of the beam will have a varying path length depending on the exact position of the moving mirror relative to position L, this value is defined as D (Figure 2:3). The path length travelled by the second half of the beam is therefore 2(L + D) [77].

The two halves of the beam recombine at the beam splitter to form an interference pattern due to the difference in path length between the two beams. This interference pattern is called the interferogram, at the centre of which is the centre burst with a series of waves distributed symmetrically either side of it [77]. The centre burst occurs were the path length difference between the two beams is zero, at this point the maximum amount of radiation passes through the interferometer since this is the positive interference at every frequency. The maximum amount of radiation directed on to the detector is however only ~50% of the initial source power [77]. The interferogram is the IR spectral information shown in the time domain and as such not straight forward to interpret, by performing a Fourier transform however the information is converted from the time domain in to the frequency (or commonly wavenumber) domain. In this form the chemical information from the sample is clearly represented and the spectra are therefore much easier to analyse [77].
2.1.2 Fourier transform Infrared microspectroscopy

In order to analyse small biological samples such as cells and tissues it is more favourable to used microspectroscopy as opposed to the macrospectroscopic technique previously discussed, although work has been done previously using conventional spectroscopy [78]. Microspectroscopy is more favourable for the analysis of these types of biological samples because it is possible to achieve greater spatial resolution, compare histological data with the IR spectra and even generate images of the tissue based on its chemistry without the need for staining thanks to the motorisation of the microscope stage [2, 24, 79]. This technique uses an infrared spectrometer coupled to a microscope. The microscope itself is similar to a standard light microscope except that the mirrors are either able to reflect both IR and optical light or there are two different sets of mirrors that are interchangeable depending on which source of light is required [79]. The earliest IR microscopes were pioneered by Barer et al. in the late 1940s however these proved difficult to use as the microscope and spectrometer were not coupled together [80]. It was not until the mid to late 1980s that the first FT-IR microscope instruments became available with some of the earliest studies being on wheat kernels and red blood cells [79]. In more recent years research has been undertaken into the possible use of FT-IR as a diagnostic method for various cancers such as prostate cancer [2, 24], melanoma [81-82], colon carcinoma [81, 83], cervical cancer [84-85], lung [37, 86], leukaemia [63] and breast cancer [87-88].
FT-IR microspectroscopy in biomedicine is mainly performed in one of two modes, transmission or transflection. In transmission mode the IR beam passes through the sample and the substrate before hitting the detector and generating the spectrum. This means the substrate used for this type of analysis must be IR transparent; CaF$_2$ is a commonly used substrate for this mode (Figure 2:4). Transflection mode differs in that the substrate used is an IR reflective material so the IR beam passes through the analyte and is reflected back off the surface of the substrate passing through the analyte again before then reaching the detector (Figure 2:4). The most commonly used substrate for transflection FT-IR is the MirrIR slide.

![Diagram of the two mainly used FT-IR modes.](image)

Recent research however has cast doubt on data obtained in transflection mode. The problem is that the electric field standing wave effect means that the strength of the electric field is dependent upon the height above the reflecting surface. Since this will vary from cell to cell, spectral differences will be introduced that are not due to changes in chemistry. For this reason it is no longer recommended as a reliable method for obtaining infrared spectra of single cells [89-92].
2.1.3 Synchrotron Radiation based (SR) Fourier transform Infrared microspectroscopy

2.1.3.1 What is synchrotron radiation?

SR is electromagnetic radiation emitted from charged particles as they are subject to acceleration. In today’s 3rd Generation Light Sources dedicated to SR research, magnetic fields are applied via bending magnets to hold electrons in a closed orbit: the centripetal acceleration given by a vertical magnetic field is the source of the SR emitted tangentially in bending magnet beamlines. The rate of acceleration experienced by the charged particle affects the intensity of the SR emitted such that the greater energy of the stored electron the greater the SR power emitted becomes [41, 93]. SR is emitted simultaneously over a wide frequency range from the far-IR to hard X-rays and as such synchrotrons are referred to as extremely broadband sources [41]. Due to the relativistic effect on the orbit of electron circulating close to the speed of light, the emitted SR fan is also highly collimated in angle and originating from a micrometric electron current, plus the emission is intrinsically polarised in the electric field [41, 94]. In the specific of IR radiation, the figure of merit of SR is the photon flux density emitted per unit area of the source and per angle of emission: this parameter called brightness and in the mid IR region a SR is 100 to 1000 times more bright than a conventional broadband source [95]. In practice this parameter is the measure of the number of photons per second that can be refocused onto a micrometric sample by a microscope. It is this brightness of SR-IR that allows i) illuminating small objects comparable in size to the wavelength (diffraction limited microbeam), ii) resolving IR spatial images at the highest optically attainable resolution (diffraction limited spatial resolution), and iii) obtaining FT-IR spectra in a few seconds with a suitable spectral quality (Signal to Noise ratio) not achievable by conventional thermal source [40, 96]. The most commonly used thermal source is the globar i.e. a blackbody emitter maintained at temperature which it glows orange and maintained by an electrical current [55].
2.1.3.2 The structure of a synchrotron

Nowadays synchrotrons are large facilities allocating the simultaneous work of many laboratories, called beamlines, which perform experimental research simultaneously. For example the Diamond Light Source at Didcot in the UK has a circumference of 561m and houses 22 fully operational beamlines with another 10 beamlines currently planned, under construction or in final testing [97]. A modern synchrotron radiation facility consists of five main parts, an electron gun, a linear accelerator (Linac), a booster ring, a storage ring and the beamlines [93, 98]. The exact arrangement of these parts, as well as the design of the storage ring can vary. Here the UK national facility Diamond Light Source located at Didcot will be used as an example for further discussion (Figure 2:5).

Figure 2:5 Schematic of the synchrotron at Diamond. The linac, booster ring, storage ring and endstations are labelled. Electrons flow from the linac to the booster ring and into the storage ring. The SR is then focused down the beamlines and in to the instruments on the endstations [97].

The electrons which generate the SR are produced in an electron gun. The electrons are produced by heating a metal filament under vacuum to a temperature at which electrons are liberated from the metal surface. This process is known as thermionic emission. These electrons are then accelerated until they reach 90 kilo electron volts (keV) at which point they pass into the linac. The linac houses a series of radiofrequency (RF) cavities, these units further accelerated the electrons
to a relativistic energy of 100 mega electron volts (MeV). The electrons are also grouped into bunches within the RF cavities [94, 97-98]. The linac also contains focusing elements that are used to reduce the size of the electron beam which increases the brightness of the SR emitted by the synchrotron [94]. The final element of the injector system is the booster synchrotron which is a combination of RF cavities and bending magnets [94]. The booster synchrotron at diamond follows a stadium shape (Figure 2:5). The electrons are accelerated in the straight sections by using RF units and guided round the bend using 36 dipole bending magnets [94, 97]. The electrons are injected into the storage ring once they reach an energy level of 3 giga electron volts (GeV) [97]. The storage ring is where the SR is generated. The storage ring is a complex arrangement of several different components including, bending magnetic, RF units and insertion devices [94, 97]. The most important fact about the storage ring is that it is not a circular structure but a polygon. The electron beam is curved round the bends by bending magnets; the electrons therefore undergo a large orthogonal acceleration which results in the production of intense SR [41]. The SR used for the B22 IR beamline at diamond is generated in this way [97]. The other method of generating SR within the storage ring is by the use of insertion devices. There are two main types of insertion device: wiggler and ondulators. The first is used to boost the total photon flux at in a broad spectral range up to hard X rays. The second emits a narrow band of radiation (plus harmonics) with enhanced brilliance [97, 99]. These insertion devices are placed on the straight sections between the bending magnets prior to the beamline which requires their use [99]. Spectroscopy beamlines typically prefer bending magnet given their reliability and broadband character and this is the case of IR beamlines [99].The final component of the storage ring is the RF system. This is a series of RF units placed around the storage ring. The purpose of these RF units is to replace the energy the electrons lose after they have emitted the SR [41].
The last element of the synchrotron is the beamline. This is where the SR emitted from the storage ring is focused into the analytical instruments for sample analysis. As most of the SR work performed during this project was carried out on B22 at the Diamond light source the hutch layout and optics arrangement used for this beamline are used as an example. The hutch and optic layouts for this beamline are shown in Figure 2:6.

![Figure 2:6 a) layout of the experimental hutch of B22 at Diamond Light Source [97]. b) layout of the focusing optics used in the B22 beamline at the Diamond Light Source [97].](image)

The beamline can be thought of in two parts, half inside the synchrotron tunnel and half in the experimental cabins where the instruments are housed and the final focusing of the SR beam occurs; in the case of B22 a sample preparation area is also present (Figure 2:6 a)). The second part of the beamline is the optics which focus and direct the SR from the bending magnets in the storage ring on to detector of the IR microscope at the end of the beamline (Figure 2:6 b)) [97].
The B22 beamline endstations at the Diamond light source use the Bruker Vertex 80v in-vacuum-FT-IR coupled to a Hyperion 3000 IR microscope (Figure 2:7).

![Figure 2:7 Photo of one of the endstations at the B22 beamline.](image)

### 2.1.3.3 Conventional SR-FT-IR microspectroscopy

There are two main forms of conventional SR-FT-IR study. The first is in the collection of single whole cell spectra using aperture sizes equivalent to that of the cell diameter, usually between 10 and 20 μm. One set of sampling parameters commonly used for this type of study is 256 co-scans at 4 cm⁻¹ spectral resolution as demonstrated by Hughes et al., Flower et al., Raab and Martin, and Solomon et al. [29, 35, 100-102]. In order to improve spatial resolution the spectra can be collected in a confocal arrangement. This uses an aperture both before and after the sample in the IR beam path and results in an improvement of ~30% in spatial resolution [103]. By using confocal apertures of 7 x 7 μm and step sizes of 3 x 3 μm it has been shown that it is possible to obtain chemical maps of single cells with subcellular spatial resolution [27, 104-105]. This is however a very time consuming process especially with larger cells such as the cells of the prostate cancer cell line PC3 which can be up to 30 μm in diameter [3]. It is therefore common for studies which require the collection of very large quantities of FT-IR spectra such as this kind of cell mapping to use 128 co-scans as this significantly reduces the time required to obtain each spectrum and therefore significantly reduces the overall data acquisition time [27, 106]. However even using only 128 co-scans, the time required to collect a spectral map of a large PC3 cell would be significant. For example in order to perform a single cell mapping using conventional SR-FT-IR such as Gazi et al. on a 30 μm PC3 cell it would require the collection of a total of 100 sample
spectra and 10 background spectra; as the collection of a spectrum at 128 co-scans takes ~2 mins the total acquisition time of a single cell map was ~3.6 hrs [27].

2.1.4 Focal Plane Array FT-IR imaging

FT-IR imaging achieves spatial resolution by dividing the detector in to an array of smaller elements as opposed to other FT-IR techniques which close the aperture size to increase spatial resolution [40, 107]. This array of detector elements is arranged into a square grid structure, within which each individual element collects a single FT-IR spectrum (Figure 2:8). The collected spectra are arranged in to a single spectral image providing a multiplex advantage over other single cell FT-IR techniques. This multiplex advantage can lead to a reduction in data acquisition times. For example to collect 100 single cell spectra using 4 cm⁻¹ resolution and 256 co-scans, requires ~3 mins per spectrum as well as the collection of a background spectrum every 5 spectra. The total data acquisition time is therefore 6 hrs. It is possible to obtain ~300 single cell spectra in a single map using a FPA from a well prepared sample. As the time taken to acquire a single background and map at 4 cm⁻¹ and 512 co scans takes 1.5 hrs, the time saved is significant.

It is important to note however that in order to gain a significant advantage over the SR-FT-IR the sample preparation is important. This is because if the cells are too far apart then only a few will be collected per image and SR-FT-IR may prove to be the more efficient method of obtaining single cell FT-IR spectra.

![Illustration of a FPA detector. The detector array is shown in blue with each separate element separated by a black line. An example FT-IR image is shown to represent what the spectra map looks like and finally a single extracted spectrum is shown.](image)
The FPA system used in the data collection for this thesis was a Varian 620-IR FT-IR spectrometer coupled to a Varian 620-IR FT-IR microscope with a 128 x 128 focal plane array pictured in Figure 2:9.

![Photo of the Varian FT-IR imaging system](image)

Figure 2:9 Photo of the Varian FT-IR imaging system

### 2.1.4.1 SR-FPA-FT-IR imaging

A recent development in the field of FT-IR imaging is the coupling of FPA systems to a Synchrotron source (SR-FPA-FT-IR imaging). This combination of detector and source was first developed in 2006 by three groups independently of each other [108-110]. However, they all used a single beam to illuminate the FPA. The result of using a single SR beam was a localised increase in brightness corresponding to the focal point of the SR beam on the FPA detector. Although the region of the FPA illuminated by the SR beam does yield greater SNR than that of a standard thermal source, the spot size of the SR beam severely limits the maximum size of FPA that can be used. The only other way of compensating for the heterogeneous illumination is to increase the integration time which leads to a decrease in the acquisition speed, which is one of the main advantages of using an FPA [111].
This problem has however been solved by the Infrared environmental imaging (IRENI) beamline at the Synchrotron Radiation Centre synchrotron facility in Wisconsin USA. They achieved this by using a large fan of radiation from a bending magnet which is then split into 12 separate beams. These beams are then arranged into a 3 x 4 bundle capable of illuminating the whole of their 96 x 96 pixel FPA detector (Figure 2:10) [111].

![Figure 2:10](image_url)

**Figure 2:10** a) schematic of the main mirror positions at the IRENI beamline. b) long exposure photograph of the beam bundling between mirrors 3 and 4 [111-112].

The combination of this detector with a high numerical aperture (0.65) x 74 objective, allows imaging of single cells and small tissue areas (54 μm x 54 μm) at a pixel resolution of 0.54 μm [113]. This is below the diffraction limit achievable using a conventional confocal FT-IR microscope configuration and the highest numerical aperture objectives commercially available which is 0.6 μm [111]. Although this instrumentation is excellent for high detailed spectral analysis of cells and tissues, the working distances and data collection times are too small and too long to be applied to the through-put device designed and constructed during this project.
2.2 Atomic force microscopy (AFM)

AFM is a commonly used technique for analysis of surface morphology [114]. The AFM used here was a multimode picoforce AFM manufactured by Digital instruments (Figure 2:11 a)). The instrument can be thought of as three parts; the head unit, the AFM chip and the base. The base houses the laser power supply and provides a digital read out of the current force measurement. A stage is also located above the base which allows the sample to be moved during AFM imaging data collection. The second component of this AFM is the head unit. This is where the mirrors and laser that are used to measure the deflection of the laser beam are housed. The final part of the AFM is the ‘AFM chip’. A picture of one of the chips used to collect the data is shown in Figure 2:11 b). The bulk of the chip is there only to allow accurate placement of the tip in relation to the sample. The most important part of the chip is the cantilever on to which the laser is focused and it is the bending of this cantilever and the subsequent deflection of the laser beam that is used to calculate the forces experienced by the tip. The final part of the chip is the tip; this is a tiny needle-like structure which is moved across the sample surface. As the tip is scanned across the surface of the sample it is pulled towards the sample surface by intramolecular forces, the further away the surface the more the cantilever will bend and the closer the cantilever is to the surface the less it bends [115]. If a laser is aimed at the point of the cantilever directly above the tip, the reflected laser beam will be deflected as the tip is scanned across the sample surface. This deflection can then be used to calculate the surface topography. AFM can be performed in air or under vacuum as well as in contact or non-contact mode [116].

Figure 2:11 a) picture of the multimode picoforce AFM and b) a close up of the AFM chip
2.3 General data analysis methods

2.3.1 Data analysis

The data analysis methods used during this project were the same for both the data collected using a synchrotron and that obtained using lab based FT-IR imaging system in Manchester. The only exception is that the single cell data collected using FT-IR imaging must first be extracted from the spectral image. This is done using an in house function, in which a threshold value for the amide I spectral region is used to isolate the image pixels which contain cellular spectra. The spectra for each individual cell are then averaged together to produce a single spectrum for each cell.

2.3.2 Mie scattering

Mie scattering is a form of light scattering that results when small particles are illuminated with electromagnetic radiation at a wavelength similar to the size of the particle under observation. This is important in FT-IR as the wavelengths of radiation used range between 1-10 μm; this is similar to the diameter of cell organelles such as the cell nucleus and mitochondria [4, 117]. This form of scattering is particularly problematic for FT-IR analysis as the absorbance bands detected for different chemical species can have their peak position shifted and the shape of the absorbance band itself can become distorted due to this scattering effect. As both the shifting of the peak position and changes in the band shape are commonly associated with changes in cell biochemistry the problem of Mie scattering is a major issue in the application of FT-IR in biospectroscopy [34, 118].

Mie scattering is a function of both changes in refractive index and the size of the scattering particle from which the scattering results [118-119]. The Mie scattering that is present in FT-IR spectra obtained from cell and tissues is further complicated because these are absorbing particles whereas the original Mie scattering approximation only describes non-absorbing particles. The fact that the scattering particles in cells are absorbing particles is important because the refractive index of the particle changes across the absorption band, for this reason this form of Mie scattering is referred to as resonant Mie scattering [34, 118]. In order to fully explain
the resonant element of the Mie scattering observed in the FT-IR analysis of cells it must be understood that the refractive index is composed of two parts \( n \) and \( k \) as described in Equation 2-3 [118].

**Equation 2-3** \[ n = n + ik \]

Where \( n \) is the total refractive index, \( n \) and \( k \) are the real and imaginary elements of the refractive index [118].

The variation in the two elements of the refractive index across an absorption band is described by the Kramers-Kronig equation. The resulting variation in the two elements of the refractive index across a simulated absorbance band is demonstrated in Figure 2:12 [118, 120-121].

![Figure 2:12 Calculated change in the real (n) and imaginary (k) components of the refractive index [122].](image-url)
The changes in peak position associated with Mie scattering are therefore due to this change in the real refractive index component across the absorption band and the effect this has on the scattering coefficient (Q) as shown in Equation 2-4 [122].

\[ Q = 2 - \left( \frac{4}{\rho} \right) \sin(\rho) + \left( \frac{4}{\rho^2} \right) [1 - \cos(\rho)] \]

Where \( \rho = \frac{4\pi d(n-1)}{\lambda} \) and \( n = \frac{n_1}{n_2} \)

The \( n_1 \) and \( n_2 \) in Equation 2-4 refer to refractive indices of the sample and the surrounding medium [122]. Although a correction algorithm to remove this form of scattering has recently been developed referred to as the RMieS-EMSC scattering correction, it is still worthy of note that if the refractive indices of \( n_1 \) and \( n_2 \) can be matched then no Mie scattering will be observed [34, 118, 122-123].

The Mie scattering commonly encountered during FT-IR analysis of fixed cell samples in air occurs as a result of the difference in refractive index between the biological sample (which is 1.3) and the refractive index of air (which is 1) [124]. However the refractive index of water is also 1.3 and therefore is a refractive index match for biological samples [125]. The collection of aqueous cell spectra would therefore remove the need to correct for Mie scattering because without a difference in refractive index Mie scattering cannot occur.

### 2.3.3 Vector normalisation

Vector normalisation is a process commonly used in the analysis of FT-IR spectral data. The purpose of the process is to remove absorbance differences which are caused by changes in thickness of the analyte. Vector normalisation scales the data according to Equation 2-5.

\[ \]
Equation 2-5 \[ \sum_k (A_k - \bar{A})^2 = 1 \]

Where \( A_k \) is the absorbance value at \( k \) cm\(^{-1} \) and \( \bar{A} \) is the average absorbance value of the data set in same spectral region [126].

### 2.3.4 Principal Component Analysis (PCA)

PCA is an unsupervised data analysis method which works by deconstructing a data set into a series of principal components (PCs). Each PC accounts for a percentage of the total variation in the data set, with the PC describing the largest amount of variance assigned to PC1. In order to do this the data is first broken down into two matrices referred to as the scores and loading matrices (Figure 2:13) [127-128].

![Illustration of the PCA method](image)

Figure 2:13 Illustration of the PCA method [127]. A is the number of PCs needed to fully describe all of the variation in the data set.

PCA is used in this project as a clustering method. This is done by plotting PCs against one another to generate a scores plot which allows visual interpretation of the data. When the different data sets can be clustered separately, the analysis of the loading vectors corresponding to the PC axes used, to generate the relevant scores plot allows the factors governing the greatest variance between the groups.
to be determined. In the case of FT-IR analysis the easiest way to analyze to loading data is to plot the vector along a wavenumber axis, the differences in variance at each wavenumber can then be correlated to changes in cell chemistry. This process is best demonstrated using an example such as Hughes et al. 2012 [100]. The aim of the paper was to investigate the cellular response of a renal cell carcinoma cell line (Caki-2) to novel chemotherapeutic agents. Figure 2:14 shows the scores and loading plots of the spectra collected from cells incubated with the drug KF13 for 3 days compared with spectra of untreated control cells. The scores and loading plots are shown as a) and b) respectively (Figure 2:14).

Separation between the KF13 treated and control cells can be seen along the axis defined by PC2 in the scores plot in Figure 2:14 a). The biochemical differences between the samples can be determined from the loading data when it is plotted along the wavenumber range used for the data analysis. This plot is defined as a loading plot and is shown in Figure 2:14 b). The differences in variance at each wavenumber value in the analysed range are plotted. A peak in the variance which occurs at a wavenumber value that can be attributed to a vibration of a biological compound can then be said to be as a result of changes in biochemistry between different samples in the data set [100].

Figure 2:14 a) represents the PCA scores plot comparing the drug treated (blue triangles) and untreated control (black stars) cells after 3 days of drug treatment. The accompanying loading plot is shown in b) [100].
2.3.5 Linear discriminate function analysis (LDA)

The form of LDA used for this project is the Fischer Discriminant analysis. LDA is defined as a supervised data analysis technique because the analysis takes into account prior knowledge about the data. For example when the data is collected from different cell lines the spectra obtained are labelled as belonging to one cell line or the other. This data is expressed as a class variable and used to determine the intra-class variance as well as the inter-class variance. The LDA algorithm then determines a linear boundary between the data sets and attempts to maximise the inter-class variance and minimise the intra-class variance. This can be performed as a follow up analysis to PCA in PC-LDA. This allows more information about the class separation to be obtained than simple PCA as many PCs can be condensed into a single discriminate function (DF). The DF is calculated by taking the cosine vector which best describes the separation in two PCs as shown in Figure 2:15.

![Figure 2:15](image)

Figure 2:15 a) shows an example of a two class problem where both PC axes describe some of the separation. b) illustrates how by rotating the axes a vector can be generated which fully describes the separation between the classes. This vector is referred to as DF1 [129].

This process is repeated for each combination of the nominated PCs entered into the LDA i.e. PCs 1-5, the resulting cosine vectors are then combined to give the vector that best describes the separation of the data. This is something of a ‘double edged sword’ however, as by using more PCs it is possible to introduce noise to the separation variable and so over fit the data [127-128].
CHAPTER 3  Development of the microfluidic device

The device developed for this project had to fulfil a number of criteria. The first of these was that the device had to be able to transport a cell from an input reservoir to a point at which the cell could be probed using FT-IR and finally deliver the cell to an output reservoir. This process had to be carried out reasonable quickly in order for the cells to arrive at the point of analysis alive. The analysis chamber had to have a depth of less than 10 μm, in order to prevent saturation of the detector occurring in spectral regions corresponding to the strong water absorption bands. The surfaces of the analysis chamber also had to optical flat in order to prevent light scattering phenomenon. A second reason for the analysis surfaces to be flat was any ridges and obstructions would help the cells passing through the system to adhere to the surface and in a flow system this would not be advantageous.

3.1  Initial design of the device

The device can be thought of as two distinct sections: the inner section and the outer section. The inner section is comprised of two IR transparent substrates secured one on top of the other (Figure 3:1a)). The two substrates are separated by a gap of <10 μm. However, the entire channel cannot be this thin as a single cell is larger than this distance and prolonged compression of the cell may result in changes to the cell’s biochemistry. One example of this is demonstrated by Birarda et al. who studied the response of monocytes to compression forces and found that such changes in compression forces can indeed induce changes in cell biochemistry [52]. The use of an extended thinner channel would also increase the chances of cells adhering to the upper and lower surfaces of the channel which could cause blockages and therefore prevent the cells reaching the analysis chamber. For these reasons increasing the depth of the inlet and outlet channels was preferable. This did however mean that the analysis plateau would be significantly higher than the floor of the rest of the channel. In order to reduce the mechanical stress experienced by the cells it was decided to bridge this gap using a ramp with an shallow incline of 10° (Figure 3:1b)).
Figure 3:1 a) close up of inner section of the device featuring how the two IR substrates are secured within the device. b) close up of the FT-IR analysis chamber without the upper substrate in place.

The main features of the outer section in the initial design of the device were the horizontally mounted inlets and outlet denoted as a) and b) respectively in Figure 3:2. The inlets and outlet are mounted horizontally in order to reduce the working distance between the device and the microscope objectives used for data acquisition. One other feature worthy of note was the removal of a 30° cone from the lower surface of the outer section surrounding the inner section. This was done to insure that the outer section of non-IR transparent material did not affect the FT-IR data collected using the device. The other important feature of this section of the device is the hydrodynamic focusing region, denoted as c) in Figure 3:2.

Figure 3:2 schematic of the initial design of the device. The inlets, outlet and hydrodynamic focusing region are labelled as a), b) and c) respectively.
Hydrodynamic focusing is a commonly used method for creating a continuous stream of particles or in this case, cells. Hydrodynamic focusing occurs when the sample stream is injected into a stream of sheath fluid. This causes the sample stream to narrow in the dimension of the sheath flow and so focuses the sample stream into a smaller cross section [130]. This concept is illustrated in Figure 3:3.

![Figure 3:3 illustration of hydrodynamic focusing.](image)

Hydrodynamic focusing only occurs however in systems where the flow is considered to be laminar. Fluid flow occurs as one of two states, the flow is either laminar or it is turbulent. The type of flow is characterised by the Reynolds number such that, a Reynolds number of above 2000 is considered to be turbulent whereas below 2000 is considered to be laminar [130]. The Reynolds number is a dimensionless ratio of the inertial forces which are applied to the fluid to drive it through the device and the viscous property of fluid which determines how easily the fluid flows [131]. The Reynolds number (Re) can be calculated from the hydraulic diameter (Dh) of the microfluidic device. This is done using a combination of Equation 3-1 and Equation 3-2.
Equation 3-1 \[ D_h = \frac{4A}{P} \]

Equation 3-2 \[ Re = \frac{QD_h}{\nu A} \]

Equation 3-3 \[ \nu = \frac{\mu}{\rho} \]

Where \( Q \) is the volumetric flow rate, \( A \) is the cross-sectional area of the channel, \( \nu \) is the kinematic viscosity. \( \nu \) is calculated using Equation 3-3 were \( \rho \) is the fluid density, and \( \mu \) is the dynamic viscosity of the fluid [130].

In the case of this device however where the channel width is much greater than the channel depth then the hydraulic diameter term is replaced with the value equivalent to twice the distance between the parallel plates [132]. The \( Re \) for this device is therefore calculated using Equation 3-4 were \( L \) is the depth of the channel.

Equation 3-4 \[ Re = \frac{Q2L}{\nu A} \]

The \( Re \) of this device therefore ranges from 0.1188 at the analysis chamber to 4.158 \times 10^{-4}. This means that throughout the device the fluid flow is characterised as laminar.

The method of fluid flow used for this device is pressure driven flow. The fluid is pumped from a reservoir either by using gravity or as in this case, a pump. The type of pump used with this device was a syringe pump. Initially the type of syringe pump used was a Kloehn syringe pump however these pumps require in house software to control the flow which was designed for continuous flow configurations. This led to difficulties in halting the cells in the analysis chamber and as such the type of syringe pump employed was changed to a Cole-parmer 74900 series syringe pump. These pumps incorporate an on board control system which allowed a much more responsive control of the fluid flow.
3.2 Selection of materials for the components of the device

3.2.1 The analysis chamber

The main function of the inner section is to allow FT-IR data to be obtained from cells held on the analysis plateau. The most important property of the material used for this section is therefore its transparency to IR. The most commonly used substrate materials are shown in Table 3.1. The best material to use with regard to IR transmission would be BaF$_2$, however as the water solubility of this material is low and given the toxicity of Barium ions this is not a feasible option for this device [54]. Diamond would also be a good choice as the IR transmission is reasonable and it is insoluble in water. Unfortunately diamond windows at present are extremely expensive and due to the chemical inertness and hardness of the material it is not suitable for micromachining as well as many microfabrication techniques. The material chosen for the IR windows in this device was therefore CaF$_2$ as this material is softer than diamond but more insoluble than BaF$_2$. The IR transmission profile of CaF$_2$ is also very good in the regions required for biological sample analysis. The cost of CaF$_2$ is also acceptable.

<table>
<thead>
<tr>
<th>Name of material</th>
<th>Solubility in water (g/100g of water)</th>
<th>IR transmission range (cm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaF$_2$</td>
<td>0.00151 (20 °C)</td>
<td>66666 – 1110</td>
</tr>
<tr>
<td>BaF$_2$</td>
<td>0.12 (25 °C)</td>
<td>50000 – 770</td>
</tr>
<tr>
<td>ZnSe</td>
<td>Insoluble</td>
<td>10000 – 500</td>
</tr>
<tr>
<td>Si</td>
<td>Insoluble</td>
<td>10000 – 1540</td>
</tr>
<tr>
<td>Ge</td>
<td>Insoluble</td>
<td>5000 – 600</td>
</tr>
<tr>
<td>Diamond</td>
<td>Insoluble</td>
<td>45450 – 2325</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1665 – 1110</td>
</tr>
<tr>
<td>Sapphire</td>
<td>Insoluble</td>
<td>50000 – 1780</td>
</tr>
<tr>
<td>Fused silica</td>
<td>Insoluble</td>
<td>50000 – 2500</td>
</tr>
</tbody>
</table>

Table 3.1 list of commonly used IR substrate along with corresponding information on their water solubility’s and IR transmission ranges [54].
3.2.2 The outer section

One of the disadvantages of CaF$_2$ is that it is a very brittle crystal which is therefore difficult to machine. While it was possible to machine straight channels into CaF$_2$ substrates such as those found in the analysis chamber, the side inlets and outlet used in the outer section would not be machinable. This is because the 1mm CaF$_2$ substrates required for the analysis chamber would be too fragile to be used for this machining procedure. A different material was therefore required for the construction of the outer section. The material chosen was poly (methyl methacrylate) (PMMA) as it is both low cost and routinely used for the microfabrication of microfluidic devices.

3.3 Manufacture of the analysis chamber

3.3.1 Etching the analysis chamber

The first challenge in constructing a device of this kind was how to create the analysis chamber. The first technique tried was wet etching of the CaF$_2$ using saturated aqueous Fe(NH$_4$)$_2$(SO$_4$)$_2$ solution as described by Pan et al. in 2004 [133]. Wet etching is the use of a liquid which erodes the surface of the substrate at a constant rate. The depth of the etch is determined by the length of time the etchant is applied to the substrate surface [134]. The choice of etchants for CaF$_2$ is however very limited because of the chemical stability of the CaF$_2$ crystal structure. Unfortunately the etched surface produced from the wet etching protocol was very uneven with crystal shaped protrusions and regular groove patterns commonly observed (Figure 3:4).
Figure 3: 4 images of the etched channel surface depicting the protrusions and etching lines commonly seen on the surface. All images were obtained at x20 magnification and 0.53 μm pixel resolution.

The technique was therefore deemed to be unsuitable for the function of this device as these obstructions and grooves would provide a better surface for cells to adhere to. This could therefore lead to a build-up of cells in the channel and cause the device to become blocked.
3.3.2 The use of a spacer to create the analysis chamber

A second method to generate the correct channel depth within the analysis chamber is to insert a spacer between the two CaF$_2$ substrates. The use of a spacer means that the polished surfaces on the commercially sourced discs remains untouched, meaning the upper and lower surfaces of the analysis chamber can be considered smooth. This should therefore help to limit the adhesion of cells to the device during sample delivery and analysis. A schematic diagram demonstrating this modification to the design is pictured in Figure 3:5.

![Figure 3:5 Schematic of device with spacer (yellow) between the two CaF$_2$ substrates (dark grey).](image)

3.3.2.1 Photoresist and photolithography in the manufacture of the analysis chamber

The next problem encountered in the manufacturing of the analysis chamber was finding a suitable material from which to construct the spacer. In microfluidic devices it is common to use thin plastic films for this purpose. As the channel design for the analysis chamber is a simple straight line it was possible to cut the film to the required shape. The pieces of film were then sandwiched between the CaF$_2$ plates.
and glued in to place. However it proved impossible to generate a small enough channel depth using this combination of materials. One other class of materials commonly used for this purpose are photoresists. Photoresists are, as the name suggests compounds which are sensitive to light and are used to protect a substrate during chemical etching processes. They also allow the production of precise patterns in microfluidic devices using photolithography [135]. Photolithography can be defined as the process by which a pattern is transferred from a mask to the photoresist. The photoresist may then act as a mask for further etching of the substrate beneath [136]. There are two methodologies by which this process can be accomplished. This is because there are two classes of photoresist, positive and negative. This classification refers to the effect of exposure to light on the polymerised structure of the photoresist. In a positive resist (such the one used in this project) the crosslinking between the monomeric units is weakened by exposure to the light source allowing the resist to be removed more easily by the developer solution. In a negative resist the opposite is true and the developer is used to remove the areas not exposed to the light source [134, 137]. The specific type of photolithography used in this manufacturing protocol is known as UV lithography, this is because the photoresists used in this project where sensitive to electromagnetic radiation with wavelengths in the UV region. This technique is currently used in the manufacturing of advanced microprocessors and memory chips [137]. The technique involves coating the substrate with a photoresist film which is usually done using a spin-coater. The substrate and photoresist are then pre-baked which causes the resist to polymerise and harden. A mask is then placed over the photoresist. This mask is a stencil depicting the desired channel pattern to be etched in to the photoresist. If the photoresist used is a positive resist, the channels will be defined by clear regions on a black background. In the case of a negative photoresist however the mask will appear as a black shape on a clear background [134]. The mask covered substrate is then exposed to UV light for a predetermined amount of time before the UV mask is removed. The photoresist is now ready to be developed. The photoresist is developed by submerging the photoresist covered substrate in to the developing solution again for a predetermined amount of time. The developing solution is a chemical solution which has been specifically developed to etch its partnered photoresist in a controlled way. The resulting photoresist pattern left on the substrate is then usually baked again in a process referred to as post-baking. This process increases the adhesion of the photoresist.
mask to the substrate as well as increasing the stability of the transferred pattern described by the photoresist layer. The exact pre and post baking times, the temperatures to be used as well as the spin speed, UV exposure and developer exposure times are usually provided by the manufacturer of the photoresist. For example the S1813 development protocol can be found at [138].

Photoresists have been used in combination with CaF$_2$ before such as in the work performed by Pan et al. using the Shipley S1813 resist [133]. However, when this photoresist was used to produce the spacer for this device the results were unsatisfactory. The reason for this was the poor adhesion of the photoresist layer to the CaF$_2$ substrate. This resist was therefore unsuitable for the spacer as it would have been impossible to maintain a constant channel depth and seal integrity of the analysis chamber using this material.

3.3.2.2 Use of an experimental photoresist: X AR-P 3100/10 in the manufacture of the analysis chamber

Recent work by Birarda et al. used an experimental positive photoresist X AR-P 3100/10 manufactured by Allresist in Germany designed specifically for increased adhesion [45]. The main problem with this photoresist is that, as it is an experimental photoresist it has not been well characterised. This meant that before this photoresist could be used to produce an accurate spacer for the analytical chamber a significant amount of background work had to be carried out. The first of these tasks was to determine how the thickness of the photoresist layer was affected by the speed of the spin coater i.e. how many revolutions per minute (rpm) the spin coater was set to. The second task was to determine the best exposure time of the photoresist to UV light and thirdly how long the developer solution took to remove the photoresist from the channel. Information was however provided on pre and post baking times procedures. The recommended pre-bake cycle was 85 °C for 25 minutes (mins) although only a temperature was provided for the post-bake which was between 105 and 115 °C [139].
3.3.2.3 Calibration of the spin-coater spin speed used to define the depth of photoresist film layer produced

The protocol provide by Allresist for X AR-P 3100/10 states that a spin speed of 4000 rpm should yield a photoresist layer of 5 μm [139]. However the calibration for this would have been performed using substrates such as glass or silicon as these are the standard substrate materials for photolithography.

In order to generate the calibration curve used in this project, CaF$_2$ substrates were spin-coated (using a KW-4A Precision Spin Coater) at 2000, 4000, 6000 and 8000 rpm. The resulting films were then depth profiled using atomic force microscopy (AFM). The data collected for the calibration curve was performed in contact mode and at atmospheric pressure. Examples of both the friction force and depth profile images obtained are shown in Figure 3:6.

![AFM images](image)

**Figure 3:6 AFM images of one edge of the channel.** a) was obtained using the friction force output and b) is the depth output. The AFM data was obtained from a 4000 rpm spin-coated sample.

The resulting calibration curve obtained from this experiment is shown in Figure 3:7. The ideal photoresist film thickness for this project was thought to be ~5 μm. The spin coater speed required to generate a spacer film of this thickness was determined to be ~7500 rpm. This depth is a good compromise between reducing the amount of bulk water while still allowing the cells to flow through the system.
Figure 3: Calibration curve generated for spin-coater spin speed used and the resulting photoresist film thickness produced. The data was collected in triplicate with the error bars presented here corresponding to ±1 standard deviation.

### 3.3.2.4 Determination of the UV and developer exposure time

The UV and developer exposure times were varied until the photoresist was completely removed from the floor of the analysis chamber. However the UV exposure time was increased in preference to the developer exposure time because increasing the developer exposure time resulted in thinning of the photoresist layer outside the area defined as that of the analysis chamber. Although over exposure of the photoresist to UV light (Dymax UV 2000 EC developer unit) may result in the sides of the channel not being perfectly defined, this was thought to be preferable to residual photoresist being present in the analysis chamber or affecting the spacer thickness. The final protocol for the UV exposure was 20 milliwatts per square centimetre (mW/cm²) for a period of 90 seconds (s). This value was determined using a UV minder dosimeter. The substrate was then submerged in AR 300 – 26 developer solution (Allresist, Germany) for 90 s. The substrate was then washed with deionised water to remove the developer solution and finally air dried.
3.3.2.5 Improving the repeatability of the photolithography process

Initially the photomask was simply placed on top of the photoresist coated CaF$_2$ substrate within the Dymax UV 2000 EC developer unit. This however led to inconsistent placement of the channel relative to the ideal central location, through the middle of the CaF$_2$ substrate. This meant that it was impossible to standardise the outer section of the device as the channel placement had to be adjusted for every new device. In order to solve this problem a holder was constructed in accordance with the design presented in Figure 3:8.

![Figure 3:8](image)

**Figure 3:8** exploded diagram for the UV holder. The lower and upper sections are machined from black Perspex. Four metal pillars were attached around three sides to allow reproducible placement of the upper section relative to the photoresist layer. The photomask was attached to the underside of the upper section with the desired channel running through the centre of the hole in the upper section.

The holder was machined from black Perspex (Bay plastics Ltd, North Shields, UK). This holder allowed the channel to be placed consistently and therefore the outer sections of devices to become interchangeable. This reduced the cost and time required to produce new devices, as if during the manufacturing of the analysis
chamber, this section of the device failed, it was not necessary to reproduce both section of the device for subsequent devices.

### 3.3.3 Sealing the analysis chamber

The final challenges faced in the construction of the analysis chamber were how to join the upper and lower CaF$_2$ substrates together and seal the chamber. Initially the lower CaF$_2$ substrate was set into the outer section and the upper substrate was then placed on top. A weight was then applied to press the substrates together and an epoxy resin was applied to the outer circumference of the CaF$_2$ substrates. This resulted in the bonding and sealing of the two substrates in a single step. However initial testing of the depth of the device using this cold bonding technique by interferometry determined that the depth of the analysis chamber produced using cold bonding was much greater than expected. For example, devices produced using spin speeds of 6000 rpm and 8000 rpm resulted in measured depths of about 12 μm and 15 μm respectively. The interferometry measurement was performed using a Varian 620-IR FT-IR imaging system. The spectra were collected in single point transmission mode at 4 cm$^{-1}$ resolution with 512 co added scans (co-scans). The collected spectra are shown in Figure 3:9.

![Figure 3:9 fringing spectra collected in single point transmission mode at 4 cm$^{-1}$ resolution and using 512 co-scans. The spectrum represented in a) corresponds to ~12 μm channel depth whereas b) corresponds to ~15 μm.](image)
The depth of the analysis chamber was calculated using Equation 3-5 [75].

\[ d = \frac{m}{2n(v_1 - v_2)} \]

Where \( d \) is the depth of the chamber, \( m \) is the number of fringing peaks, \( n \) is the refractive index of the material within the analysis chamber; \( v_1 \) and \( v_2 \) are the highest and lowest wavenumbers of the spectral range over which the fringing occurs. For example Figure 3:9 a) has 6 fringes between 3840 and 1300 cm\(^{-1}\). The fringing calculation would therefore look like Equation 3-6.

\[ d = \frac{6}{2\times1\times(3840-1300)} \]

This yields the result 0.001181; however this figure is in cm. The result was then multiplied by 10000 to get a result in \( \mu \)m of 11.8 \( \mu \)m.

### 3.3.4 The use of thermal bonding to seal the analysis chamber

The fact that there was a problem with the bonding of the chamber was later confirmed when water was observed to be spreading out from the central channel over the top of the photoresist layer. In order to solve this problem a second bonding method referred to as thermal bonding was then trialled. The thermal bonding step is performed instead of the standard post-baking procedure. The main difference is that in a thermal bonding step the upper CaF\(_2\) substrate is placed in the desired location above the photoresist layer. Pressure is then applied to the upper and lower CaF\(_2\) substrates in a heated environment. In the development of this device the thermal bonding step was carried out in a Carbolite CWF 1200 oven at 120 °C for a period of 72 hours (hrs) and the pressure was applied by placing a weight on top of the upper substrate. The bonding temperature and time were determined experimentally as a set protocol for thermal bonding CaF\(_2\) and X AR-P 3100/10 has not previously been developed using the equipment available for this project.

Unfortunately during the development of the thermal bonding protocol a few issues with the analysis chamber design were encountered. The first of these was that the
use of two complete circular CaF$_2$ substrates caused the photoresist layer at the circumference to bleed in to the channel. This resulted in the inlet and outlet of the analysis chamber being sealed and rendering the analysis chamber useless. The central region of the channel however remained clear of photoresist, this indicated that the photoresist layer at the circumference of the CaF$_2$ substrate maybe thicker than the central region. To resolve this problem the upper CaF$_2$ substrate was reduced to a 6 mm x 6 mm square. One other problem encountered during the development of this procedure was that the bonding, even when successful, was inconsistent with bonding occurring on only one side of the analysis chamber a common occurrence. This occurred due to uneven weight distribution over the upper CaF$_2$ substrate caused by the centre of gravity of the weight not running through the centre of the upper CaF$_2$ substrate. The problem was solved by the construction of a holder which allowed the lower CaF$_2$ substrate to be held in position while the upper CaF$_2$ substrate was placed. In addition 5 pillars were placed corresponding to the 4 corners of the weight. These were attached to the holder such that the weight could be positioned consistently with its centre of gravity at the centre of the upper CaF$_2$ substrate. A schematic diagram of the holder is presented in Figure 3:10.

![Figure 3:10](image)

Figure 3:10 The base of the holder is polycarbonate on to which the ceramic pillars were glued using epoxy resin. The pillars were positioned relative to the hollow for the lower CaF$_2$ substrate, such that the weight’s centre of gravity would pass through the centre of the upper CaF$_2$ substrate.
Polycarbonate and ceramic were used for the production of the base and pillars respectively. These materials were chosen because they have a high melting point which was an essential property for the application of this device, due for use in a relatively high temperature environment.

The completed analysis chamber also had to be inserted into the outer section in order to complete the channel from the inlet to outlet. The decrease in the size of the upper substrate also allowed easier sealing of this interface between the two sections of the device. The seals used for this interface were a combination of self-adhesive laminar sheets which were fitted over the machined channels in the outer section. These laminar sheets ran all the way up to the edge of the inner section where the film was pressed on to the proud face of the analysis chamber and a small lip sealed to the edge of the upper surface of the analysis chamber. This film seal was supported around the circumference of the analysis chamber by an epoxy resin layer as it was at this interface in the device that the greatest amount of pressure would be experienced.
3.4 Development of the outer section design

The outer section of the device changed significantly over the course of the project. The first of these changes was a reduction in the size of the core device and the production of a holder unit. This holder was constructed according to the dimensions of the sample cavities in both the Bruker and Varian FT-IR microscope stages used for data collection in this project. The design for the holder is shown in Figure 3:11.

![Diagram of holder unit](image)

Figure 3:11 exploded diagram of the holder unit with background acquisition area. The outer section of a device is shown to illustrate how the two units fit together.

One other advantage of the introduction of a universal holder unit was that this allowed a CaF$_2$ substrate to be situated outside of the device but in a similar focus to the bottom of the analysis chamber for background spectra acquisition (Figure 3:11).
Another problem that was encountered over the course of the project was that after prolonged use, the channel depth within the analysis chamber appeared to slightly increase. The increase although very small, did result in saturation effects being seen in some of the data collected. This problem was resolved by the addition of a metal plate that can be screwed in to the holder. The increased downward pressure on the upper CaF$_2$ substrate of the analysis chamber is sufficient to counter this effect.

The final complete design of the device developed over the course of this project is presented in Figure 3:12.

Figure 3:12 exploded schematic diagram of the full device as it is currently produced.
CHAPTER 4 Preliminary fixed cell work on IR substrates

4.1 Introduction

As mentioned in chapter one it is important to test the microfluidic device to make sure that infrared spectra can be obtained from single cells and that the water correction works such that meaningful biological data can be obtained. In theory the MC65 cell line represents an ideal system to use for these preliminary measurements.

4.2 Assessing the suitability of the MC65 cell line

The tet deprivation of the MC65 cells should lead to a significant increase in the expression of Aβ peptides and as such an increase in the β–sheet Amide I vibrations, which should subsequently be visible in the spectra after water correction. In order to determine if this was going to be possible, a trial study was performed using standard fixed cells on infrared transparent CaF₂ substrate analysed using transmission FT-IR microspectroscopy. A control of tet treated MC65 cells and a sample of tet deprived MC65 cells was produced for a number of time points between 4 and 120 hrs. The first FT-IR experiment performed was a standard large aperture single point FT-IR study.

4.2.1 Single point FT-IR analysis of the MC65 cell line

The large aperture FT-IR experiment involved collecting IR spectra of groups of MC65 cells without using an aperture. This generates an average IR spectrum of all the cells within the field of view. The aim of this experiment was to establish whether or not it was possible to discriminate between the IR spectra of the control population of MC65 cells which are treated with tet (tet⁺) from the tet (tet⁻) deprived MC65 cells which should be overexpressing the Aβ peptide.
4.2.1.1 Methodology for the single point MC65 tet deprivation study

4.2.1.1.1 Cell culture

The same cell culture protocol was used for all the MC65 cell experiments. The MC65 cells were cultured in Dulbecco’s modified Eagle’s medium with high glucose (DMEM) supplemented with 10% (v/v) foetal bovine serum, 1% penicillin-streptomycin and 1% L-Glutamine. When tet was required a concentration of 1µg/ml was added to the culture medium [140].

4.2.1.1.2 FT-IR

A cell aliquot was collected at each time point for both the control and the tet deprived MC65 cells. The cells were then fixed in 4% formalin as used by Gazi et al. [31]. The fixed cells were then washed in PBS and cytopspun at 1200 rpm on to 1 mm CaF₂ substrates ready for FT-IR analysis. ~30 FT-IR spectra were collected for each sample tet deprived and companion control sample using a Varian 620-IR FT-IR spectrometer coupled to a Varian 620-IR FT-IR microscope. The FT-IR spectra were collected without using an aperture and therefore the full 700 µm x 700 µm field of view allowed by the Varian microscope was sampled. The data was obtained at a spectral resolution of 4 cm⁻¹, using 256 co-scans.

4.2.1.1.3 Data processing

The FT-IR spectra were RMieS-EMSC scatter corrected and vector normalised prior to analysis by PCA. The data analysis was performed on the fingerprint spectral region between 1000 cm⁻¹ and 1750 cm⁻¹. This spectral region was chosen for the analysis as it is the most diagnostic for protein changes in the mid FT-IR spectrum. The RMieS-EMSC is also more stable over this spectral window than the higher wavenumber lipid region which can make spectral classification more difficult when this region is included in the analysis.
4.2.1.2 Results of the single point MC65 tet deprivation study

The time points that were chosen for this single point feasibility study were 48, 72, 96 and 120 hrs. These time points were chosen because in previous work by Jin et al. the MC65 cell line was seen to exhibit significant amounts of cell death after 72 hrs of tet deprivation [141]. It was therefore thought that the best chance of detecting an increase in the β-sheet vibrations using FT-IR would be between 48 and 120 hrs. Sample spectra were collected at each time point from tet treated MC65 control samples and MC65 cells which had been tet deprived.
4.2.1.2.1 Results for the MC65 cell tet deprivation study after 48 hrs of deprivation.

The first step in the analysis of this data was to compare the mean spectra for the tet treated (control) cells with that of the cells which had been deprived of tet for 48 hrs (Figure 4:1). The mean spectra show clear differences in the amide I and amide II protein regions with the tet deprived cells exhibiting a clear increase in protein signal (1655 cm\(^{-1}\) and 1540 cm\(^{-1}\) respectively) [142-143]. There is also a difference between the two spectra at \(\sim 1396-1400\) cm\(^{-1}\); this region is usually associated with vibrations of methyl groups in proteins [86, 144-145]. The fact that this band shows a decrease on tet deprivation is interesting and hints at the need for further analysis such as PCA or LDA in order to determine if this difference is represented across the whole data set or in just a few spectra. The unusual shape and large absorbance of the tet deprived spectrum between \(\sim 1217\) cm\(^{-1}\) (usually associated with nucleic acids) and \(\sim 1170\) cm\(^{-1}\) (usually correlated to either glycomaterials or proteins) is also interesting [146-147]. The reason this difference in nucleic acid vibrations is particularly interesting is because in work performed by Sopher et al. a large increase in RNA was noted in tet deprived cells when compared to tet treated cells, although this was only done after 6 days of tet deprivation [148]. The large absorbance band at \(\sim 1170\) cm is therefore probably due to an increase in proteins. The unusual band shape would therefore be as a result of the close proximity of these two bands.

![Figure 4:1 A comparison of the mean spectrum for the tet treated control data set (red) and the mean spectrum for the tet deprived cells 48 hrs after deprivation (blue).](image)
The results for the PCA of the 48 hr tet deprivation time point can be found in Figure 4:2. The two classes are clearly separated along the PC1 axis. This is not however the full picture as PC2 appears to give a slight contribution to the separation with only tet deprived cell spectra found above 0.5 and only control cell spectra found below -1 (Figure 4:2a)).

The separation of the two classes in PC1 corresponds to vibrations occurring at ~1709 cm\(^{-1}\), ~1348 cm\(^{-1}\), ~1327 cm\(^{-1}\), ~1280 cm\(^{-1}\), 1180 cm\(^{-1}\), 1126 cm\(^{-1}\), ~1030 cm\(^{-1}\) and ~1010 cm\(^{-1}\) (Figure 4:1b)). These vibrations are usually associated with nucleic acids (~1709 cm\(^{-1}\), 1327 cm\(^{-1}\) and 1010 cm\(^{-1}\)), proteins (1348 cm\(^{-1}\), 1280 cm\(^{-1}\) and 1180 cm\(^{-1}\)) and carbohydrates (~1126 cm\(^{-1}\) and 1030 cm\(^{-1}\)) [86, 143, 146, 149-152]. By comparing the mean spectra for the two classes with that of the PC1 loading plot it becomes clear that these differences do correspond with those seen in the mean
spectra (Figure 4:3). It is however noteworthy that the protein differences do not contribute significantly to the class separation. The separation described in the PC1 loading plot also appears to follow the baseline offset between the two classes particularly between ~1090 cm\(^{-1}\) and ~1400 cm\(^{-1}\). This suggests that a 2\(^{nd}\) derivative transform of the data may be necessary in order to determine the real chemical differences between these two groups.

![Figure 4:3 A comparison of the mean spectra for the control (red) and tet deprived (blue) samples with the loading plot of PC1 (green).](image)
The differences in variation which give rise to the separation in PC2 also correspond to differences in nucleic acid (~1709 cm\(^{-1}\)) and carbohydrate chemistry (~1126 cm\(^{-1}\) and 1030 cm\(^{-1}\)) [86, 146, 152]. The comparison between the mean spectra for the control and tet deprived samples and the loading plot of PC2 also shows that the differences described in the loading plot do relate to differences in the mean spectra (Figure 4:4). These differences however do seem to be partly due to changes in the baseline between the two mean spectra.

![Figure 4:4 A comparison between the mean spectrum of the control (red) and the tet deprived (blue) samples compared with the loading plot for PC2 (blue).](image)

As the PCA results for this time point appeared to be affected by possible changes in the baseline in both data sets a second derivative transformation was performed on the data. This transformation has two advantages, the first of which is that 2\(^{\text{nd}}\) derivative transformation has previously been shown to limit the effect of baseline artefacts on data analysis [153]. This transformation also helps to highlight smaller spectral changes in weaker vibrations that can appear as ‘shoulders’ on larger absorbance bands. One example of this is the β-sheet band on the amide I which can be seen at ~1635 cm\(^{-1}\) [143]. This transformation does however reduce the SNR of the data and therefore care must be taken when interpreting the results of the analysis of 2\(^{\text{nd}}\) derivative spectra. For this reason it is common to employ a smoothing algorithm such as the Savitzky-Golay fitting algorithm as this removes the higher frequency noise contributions however it is not possible to completely remove all noise contributions [154].
A PCA of the 2nd derivative spectra was therefore performed. The scores plot for this PCA shows clear separation between the two classes (Figure 4:5 a)). The separation in this case is however based entirely on PC1 as opposed to the underivatised data where PC2 also had an influence on the class separation (Figure 4:5a) and Figure 4:2 a) respectively). The PC1 loading plot for this analysis shows that the vibrations at ~1456 cm$^{-1}$, ~1035 cm$^{-1}$, ~1020 cm$^{-1}$ and ~1009 cm$^{-1}$ contribute most to the class separation. These vibrational bands are usually associated with proteins (~1456 cm$^{-1}$), carbohydrates (~1035 cm$^{-1}$) and nucleic acids (~1020 cm$^{-1}$ and 1009 cm$^{-1}$) (Figure 4:5 b)) [146, 155-157].

Figure 4:5 A PCA of the 2nd derivative spectra. The scores plot is shown in a) with the spectra for the control sample and the tet deprived cells pictured in red and blue respectively. As the separation between the two classes is based entirely on PC1 only the loading for this PC is shown in b).
These differences are consistent with those previously seen in the underivatised data. However, due to the loss in SNR during the derivatisation process, a comparison of the PC1 loading plot with the derivatised mean spectra, for both the tet treated and tet deprived samples was required (Figure 4:6). From this plot it is possible to see clear correlations between the spectral differences in the mean spectra of the control (red Figure 4:6) and tet deprived cells (blue Figure 4:6) at ~1456 cm\(^{-1}\), 1035 cm\(^{-1}\) and 1009 cm\(^{-1}\) (highlighted in yellow Figure 4:6) with the peaks in variance described by the PC1 loading plot (green Figure 4:6). Although the difference at 1020 cm\(^{-1}\) (highlighted in red Figure 4:6) is clearly present in the mean spectra it may not be completely due to changes in chemistry as the difference in the mean spectra is based on an upward spike in the tet deprived spectrum. This could therefore be an effect of the derivatisation process.

Figure 4:6 A comparison of the PC1 loading plot (green) with the mean spectra of the tet deprived (blue) and control (red) samples. All spectra have been transformed to the 2\(^{nd}\) derivative. The loading plot has been off set in order to make the spectral comparison easier. The spectral features which correspond to the difference in variation shown in PC1 are highlighted in yellow. The spectral features which although correspond to difference in variation in PC1, but which may not be due to a difference in chemistry between the samples are shown in red.
4.2.1.2.2 Results for the MC65 cell tet deprivation study after 72 hrs of deprivation.

The mean spectra for the MC65 cell control and MC65 cells after 72 hrs tet deprivation are shown in Figure 4:7. The spectra are fairly similar, however with the absence of the large visible spectral differences in the amide I and amide II previously noted in the 48 hr time point. The other difference is that the 1217 cm$^{-1}$ and 1170 cm$^{-1}$ absorbance bands appear very similar at this time point and therefore call in to question the biological association of the large differences seen after 48 hrs of deprivation. As the mean spectra of the two classes appear very similar, a PCA was performed in an effort to determine if it was possible to differentiate between the classes at this time point.

![Graph showing mean cell spectra for control and MC65 cells after 72 hrs of tet deprivation.](image)

Figure 4:7 A comparison of the mean cell spectra for the control (red) and MC65 cells after 72 hrs of tet deprivation (blue).
The separation determined using the PCA scores plot shows that the chemistry described by PC1 and PC2 both influence the separation between the tet deprived and the tet supplemented control samples (Figure 4:8 a)). The spectral bands in the loading plot for PC1 indicate changes in the carbohydrate (~1030 cm⁻¹ and ~1135 cm⁻¹) as well as lipid (~1745 cm⁻¹) chemistry (Figure 4:8 b)). The biochemical differences defined in PC2 also show variations in carbohydrate (~1030 cm⁻¹, ~1122 cm⁻¹ and ~1145 cm⁻¹) and lipid (~1745 cm⁻¹) chemistry as key factors in separating these two classes (Figure 4:8 c)) [86, 142, 145, 158].

Figure 4:8 PCA plots for 72 hrs tet deprivation. The scores plot is shown in a) and the loading plots for PC1 and PC2 in b) and c) respectively. The control cell spectra are shown in red with the tet deprived cell spectra shown in blue.
Although the separation between these two classes in the PCA scores plot is reasonably clear, it is possible to improve this as well as gaining greater insight into which biochemical changes most affect the interclass separation. This can be done by the use of a supervised method of data analysis to build on the information gained from the PCA. To do this PC-LDA was used. The first step of the PC-LDA is to assign a class label to each data point. A number of PCs are then taken from the PCA loadings and the data points rebuilt using only this many PCs. The aim of this procedure is to remove the PCs which are not relevant to the separation or those that are mainly noise from the analysis. The LDA then defines a linear boundary between the different classes. The boundary is calculated such that the intra-class variance is minimised and the inter-class variance is maximised. The result of the analysis is plotted in the same way as PCA, in LDA however, the loading variable is referred to as a discriminant function (DF) as opposed to a PC. As the DF contains the information of several PCs it is much easier to determine exactly which bands and therefore what chemistry is responsible for the separation of in this case cell sample of the same cell line but treated differently with tet. It is important however, to avoid the introduction of noise and over fitting of the data by using too many PCs. In order to avoid this, the number of PCs was limited to a number which described up to a maximum of 95% of the variation within the data.
The PC-LDA results for the 72 hr tet deprivation study are shown in Figure 4:9. The scores plot in Figure 4:9 a) shows a reasonable separation between the two classes in the DF1 axis. It is also important to note that only the separation in the DF1 axis is real, this is because unlike PCA the number of DFs defined during the LDA is related to the number of data classes contained within the whole data set. The number of DFs that account for the separation is always the total number of classes minus 1. Therefore for this analysis only the separation described by DF1 reflects the changes in chemistry between the spectra collected from the MC65 control samples and the spectra of the MC65 samples after 72 hrs of tet deprivation.

The DF1 loading plot however only shows the same carbohydrate and lipid bands as those in the PCA at ~1030, ~1135 and ~1745 cm\(^{-1}\) (Figure 4:9 b)) [86, 142, 158].

![Figure 4:9 LDA plots; the scores plot is shown in a) with the DF1 loading plot shown in b). The control cell spectra are shown in red with the tet deprived cell spectra shown in blue.](image)
One other possible way to determine if a change in the protein chemistry could be detected using this FT-IR methodology at this time point was to perform a 2\textsuperscript{nd} derivative transformation as this would highlight the small shoulders on the amide I band which correspond to the β-sheet vibrations.

The results of the PCA analysis are shown in Figure 4:10. The PCA scores plot shows some degree of separation in the PC2 axis (Figure 4:10 a)). The loading plot for PC2 indicates that this separation is based on differences in the vibrations at ~1653 cm\(^{-1}\), ~1657 cm\(^{-1}\), and ~1685 cm\(^{-1}\) all of which correspond to protein chemistry although not β-sheet secondary structure as was initially expected [142-143, 152].

![PCA Results](image)

**Figure 4:10** The PCA scores plot for the 2\textsuperscript{nd} derivative transformed spectra, 72 hr tet deprivation study is shown in a). The separation is mainly in the PC2 axis and therefore only the loading plot for PC2 is depicted in b). The control cell spectra are shown in red with the tet deprived cell spectra shown in blue.
To see if any further chemistry could be extracted from the PC2 loading plot a comparison between the mean 2\textsuperscript{nd} derivative spectra for the control and tet deprived samples and the loading plot of PC2 from the 2\textsuperscript{nd} derivative PCA was done (Figure 4:11). From this comparison it is possible to see that there is a difference between the two mean spectra corresponding to the 1657 cm\textsuperscript{-1} seen in the loading plot (highlighted in yellow Figure 4:11). The difference in the loading plot at 1653 cm\textsuperscript{-1} however, appears to correspond to a difference between the mean spectra very near the baseline of the spectral peak at \(~1657\) cm\textsuperscript{-1} (Figure 4:11 highlighted in red). It is therefore probably the result of slight change in peak width due to the derivatisation process. The difference shown in the loading plot at 1685 cm\textsuperscript{-1} however appears to be the result of a peak shift in the tet deprived cells from 1685 cm\textsuperscript{-1} to 1694 cm\textsuperscript{-1} which could indicate an increase in the \(\beta\)-sheet content of the cells (highlighted in yellow Figure 4:11) [143, 152].

Figure 4:11 A comparison of the mean 2\textsuperscript{nd} derivative spectra for the MC65 control sample (red) and the MC65 sample after 72 hrs of deprivation (blue) with the loading plot for PC2 of the 2\textsuperscript{nd} derivative PCA. The loading plot has been off set in order to make the spectral comparison easier.
4.2.1.2.3 Comparison of MC65 cells after 96 hr of tet deprivation

The mean spectra for the MC65 control and tet deprived MC65 cells after 96 hrs of deprivation are shown in (Figure 4:12). There is very little difference between the two spectra and therefore a PCA was performed in an effort to determine if any chemical differences between the two classes could be found.

Figure 4:12 A comparison of the mean cell spectra for the control (red) and MC65 cells after 72 hrs of tet deprivation (blue).
The data for the 96 hr tet deprivation time point unfortunately did not show separation between the control and the tet deprived samples after either PCA or LDA analysis in this experiment. The PCA and LDA scores plots are shown in Figure 4:13 a) and b) respectively.

Figure 4:13 The PCA and LDA scores plots for the 96 hr tet deprivation time point are shown in a) and b) respectively. The control cell spectra are shown in red with the tet deprived cell spectra shown in blue.
As there did not appear to be any separation between the two classes at this time point in either PCA or the LDA a second derivative transform was performed on the data in an attempt to highlight smaller differences that would not necessarily be seen in the underivatised analysis. The results of the PCA for the 2nd derivative data are shown in Figure 4:14. A degree of separation between the two classes at this time point can now be seen in the scores plot (Figure 4:14 a)). The separation is along the PC2 axis for which the loading plot is shown in Figure 4:14 b). The most variance between the two classes is at 1105 cm\(^{-1}\), 1282 cm\(^{-1}\), 1525 cm\(^{-1}\), 1620 cm\(^{-1}\), 1655 cm\(^{-1}\), 1685 cm\(^{-1}\), 1694 cm\(^{-1}\) and 1716 cm\(^{-1}\). These vibrations correspond to differences in carbohydrate (1105 cm\(^{-1}\)), protein (1282 cm\(^{-1}\), 1655 cm\(^{-1}\), 1685 cm\(^{-1}\), 1694 cm\(^{-1}\)) and nucleic acid chemistry (1525 cm\(^{-1}\), 1620 cm\(^{-1}\), 1716 cm\(^{-1}\)) [142-144, 150-152, 157].

Figure 4:14 PCA results for the 96 tet deprivation study. The scores plot, shown in a) displays a degree of separation between the MC65 control (red) and the MC65 cell spectra after 96 hrs of tet deprival (blue). The separation is only in PC2 for which the loading plot is depicted in b).
A comparison of the PC2 loading plot for the 2\textsuperscript{nd} derivative PCA with the 2\textsuperscript{nd} derivative transformed mean spectra for both classes was also done (Figure 4:15). This was done to ensure that the chemistry described in PC2 related to real differences in the spectra obtained for each class. The comparison of the mean spectra with the PC2 loading plot shows that the differences highlight in PC2 at 1105 cm\textsuperscript{-1}, 1282 cm\textsuperscript{-1}, 1655 cm\textsuperscript{-1}, 1694 cm\textsuperscript{-1} and 1716 cm\textsuperscript{-1} do relate to differences in the mean spectra of the two classes (highlighted in yellow Figure 4:15). However the differences at 1525 and 1620 correspond to differences at the base of absorbance peaks in the mean spectra and may therefore have been due to noise introduced during the derivatisation process (highlighted in red Figure 4:15).

Figure 4:15 A comparison of the mean 2\textsuperscript{nd} derivative spectra for the MC65 control cell samples (red) and that of the MC65 cell samples after 96 hrs of tet deprivation (blue) with the PC2 loadings of the 2\textsuperscript{nd} derivative PCA (green).
4.2.1.2.4 Comparison of MC65 cells after 120 hr of tet deprivation

The mean spectra for the two classes are shown in Figure 4:16. Although there are clear differences between the mean spectra these appear to be the result of a shift in the baseline between the two samples. However, to determine if this shift in the baselines of the average spectra disguised the differences in cell chemistry between the two classes, a PCA was performed.

![Figure 4:16 A comparison of the mean spectra for the control (red) and tet deprived cells after 120 hrs of tet deprivation (blue).](image)
The PCA results for this time point show some separation between the two sample classes with PC1 and PC3 depicting the chemistry which gives rise to the best separation (Figure 4:17). The loading plot for PC1 indicates differences in carbohydrate chemistry (~1028 cm\(^{-1}\) and ~1136 cm\(^{-1}\)) and lipid chemistry (~1738 cm\(^{-1}\)). The loading for PC3 also describes separation based on changes in carbohydrate chemistry indicated by differences at ~1028 cm\(^{-1}\), ~1128 cm\(^{-1}\) and 1145 cm\(^{-1}\) [142, 152, 155, 159].

Figure 4:17 PCA results for 120 hrs tet deprivation. The scores plot is shown in a) along with the loading plots for PC1 in b) and PC3 in c). The control cell spectra are shown in red with the tet deprived cell spectra shown in blue.
The fact that the differences reflected in both loading plots appears to correspond to the region where the apparent shift in baseline is most evident could mean that the chemical differences described in the PCA are unlikely to be actual changes in the cellular chemistry (Figure 4:18). A 2\textsuperscript{nd} derivative transform was therefore performed on the spectra in order to remove these shifts in baseline and to highlight the smaller changes in chemistry between the two classes.

Figure 4:18 A comparison of the mean spectra for the control (red) and tet deprived (blue) MC65 cells with the loading plots for the PCA (green). The loading plot for PC1 is pictured in a) whereas the loading plot for PC3 is shown in b).
The results of the PCA for the 2\textsuperscript{nd} derivative are shown in Figure 4:19. The scores plot for the 2\textsuperscript{nd} derivative shows a degree of separation however there appears to be a difference within the tet deprived data set (Figure 4:19 a)). One possible explanation for this split in the tet deprived data set is that some of the areas sampled may have contained cells with an accumulation of the Aβ peptides whereas others may not. The loading plot for PC1 shows that the variance which best describes the separation in PC1 occurs at $\sim$1003 \text{ cm}\textsuperscript{-1}, $\sim$1485 \text{ cm}\textsuperscript{-1}, $\sim$1506 \text{ cm}\textsuperscript{-1}, $\sim$1556 \text{ cm}\textsuperscript{-1}, $\sim$1657 \text{ cm}\textsuperscript{-1}, $\sim$1670 \text{ cm}\textsuperscript{-1} and $\sim$1694 cm\textsuperscript{-1}. These wavenumbers are usually associated with proteins ($\sim$1003 cm\textsuperscript{-1}, $\sim$1485 cm\textsuperscript{-1}, $\sim$1556, $\sim$1657, $\sim$1670 cm\textsuperscript{-1} and $\sim$1694 cm\textsuperscript{-1}), lipids ($\sim$1504 cm\textsuperscript{-1}) and nucleic acids ($\sim$1556 cm\textsuperscript{-1}) [146, 152, 160].

Figure 4:19 PCA results for the 2\textsuperscript{nd} derivative data. The scores plot is shown in a) and exhibits a degree of separation. The control cell spectra are shown in red with the tet deprived cell spectra shown in blue. The loading plot for PC1 is shown in b) as the separation between the two classes is described by this PC.
As the loading plot for PC1 of the 2\textsuperscript{nd} derivative transform PCA is quite noisy a comparison of the PC1 loading with the mean spectra for each class was done to help differentiate between noise and changes in chemistry (Figure 4:20).

![Figure 4:20](image)

**Figure 4:20** A comparison between the MC65 control (red) and MC65 tet deprived (blue) mean cell spectra with the loading plot for PC1 (green).

The difference in PC1 corresponding to \(\sim1003 \text{ cm}^{-1}\) does not appear to be present in the comparison of the mean spectra (highlighted in red Figure 4:20). However, this may be due to the fact that the tet deprived spectra where clearly divided in to two groups and as such this difference described in the PC1 may have been lost during the averaging of the whole tet deprived data set. The difference shown in PC1 at \(\sim1485 \text{ cm}^{-1}\) appear to be the result of a difference in an upward peak in both mean spectra and as such may not be the result of a difference in chemistry but an artefact of the derivatisation process (highlighted in red Figure 4:20). The same reasoning can also be applied to the peaks at \(\sim1504 \text{ cm}^{-1}, \sim1556 \text{ cm}^{-1},\) and \(\sim1670 \text{ cm}^{-1}\) (highlighted in red Figure 4:20). The peaks at \(\sim1657 \text{ cm}^{-1}\) and \(\sim1694 \text{ cm}^{-1}\) however do correspond to differences between the two mean spectra and interestingly appear to show a decrease in \(\alpha\)-helical protein secondary structure (\(\sim1657 \text{ cm}^{-1}\)) and an increase in \(\beta\)-sheet secondary structure (\(\sim1694 \text{ cm}^{-1}\)) upon tet deprivation at this time point (highlighted in yellow Figure 4:20).
4.2.1.2.5 Conclusions of the single point MC65 tet deprivation study

The results of the single point study did show that a slight increase in β-sheet (1694 cm\(^{-1}\)) content within the tet deprived MC65 cell populations could be detected after 120 hrs though not in all samples. A similar increase at 1694 cm\(^{-1}\) could also be seen after 72 and 96 hrs although this was much less pronounced than after 120 hrs. Although in order to detect these changes in chemistry it was necessary to perform a 2\(^{nd}\) derivative transformation. This was unexpected as MC65 cell are known to show an increase in the production of the A\(\beta\) protein of ~10x upon tet deprivation [161]. One possible explanation for the small increase in β-sheet content detected and the fact that even this relative small increase was only seen after 96 hrs of deprivation is that the MC65 cells could have been secreting the A\(\beta\) protein. To test this theory an investigation was carried out using deprivation time points covering the first 24 hrs. As the accumulation of A\(\beta\) at these time points was likely to be a less common event than in the later time points studied here single cell synchrotron FT-IR methodology was used.

4.2.1.3 Results of the SR-FT-IR analysis

The time points chosen for the SR-FT-IR study were 4, 8, 12, and 24 hrs. These time points were chosen as SR-FT-IR experiments are performed over a limited time. In order to collect a significant data set for each time point the number of time points had to be limited. These four time points therefore offered the best coverage of the first 24 hrs whilst still keeping a small enough sample set to collect a significant number of spectra from each sample. The samples were prepared using the same cell fixation and cytopsining protocol as previously stated in 4.2.1.1.2. ~60 single cell were obtained for each tet deprived and companion control sample. These spectra were collected at 4 cm\(^{-1}\) resolution using 256 co-scans at the B22 beamline of the Diamond Light Source.
4.2.1.3.1 Comparison of MC65 cells after 4 hr of tet deprivation

The initial comparison of the mean spectra for the MC65 tet control and tet deprived samples is shown in Figure 4:21. There is a clear difference between the two spectra however it does appear that there is a shift in the baseline between the two samples and this may influence any further analysis.

![Figure 4:21 A comparison of the mean spectra at the 4 hr time point. The mean control cell spectrum is shown in red and the tet deprived spectrum in blue.](image-url)
The PCA results for the 4 hr time point indicate that even after only 4 hr the two classes are starting to exhibit slight changes in biochemistry (Figure 4:22). The changes in PC1 correspond to differences in nucleic acid (~1007 cm⁻¹), carbohydrate (~1136 cm⁻¹), lipid (~1734 cm⁻¹) and protein chemistry (~1695 cm⁻¹) (Figure 4:22). The protein band at ~1695 cm⁻¹ is particularly interesting as this corresponds to the high frequency β-sheet vibration of the amide I. [142-143, 146, 155, 162].

Figure 4:22 PCA results of the single cell spectra collected from the 4 hr time point sample. The scores plot is shown in a) and the PC1 loading plot is shown in b). The control cell spectra are shown in red with the tet deprived cell spectra shown in blue.
The fact that the main differences in chemistry appear to be at either end of the loading plot is however a concern as this may indicate that these observed changes in chemistry may in fact be due to the previously described shift in baseline between the two classes. To determine if this was indeed the case a comparison between the loading plot of PC1 and the mean spectra for each class was done (Figure 4:23). The loading plot clearly follows the differences in baseline between the two classes and therefore any conclusions drawn from the PCA about changes in chemistry between the samples must be reconsidered. In order to limit the effect of this shift in base line on the data a second derivative transform was performed.

![Figure 4:23](image-url) A comparison of the mean spectra for the MC65 control (green), MC65 cells 4 hr after tet deprivation (blue) and the loading plot for PC1 (red).
The results of the PCA for the 2\textsuperscript{nd} derivative transform are shown in Figure 4:24. The scores plot for the 2\textsuperscript{nd} derivative transform PCA show a degree of separation between the two classes (Figure 4:24 a)). The separation seen in the scores plot is along the PC1 axis. The chemistry which describes this separation is therefore described within the PC1 loading plot shown in Figure 4:24 b). The peaks which correspond to most of the variance between the two classes in PC1 occur at 1010 cm\textsuperscript{-1}, 1014 cm\textsuperscript{-1}, 1030 cm\textsuperscript{-1}, 1040 cm\textsuperscript{-1}, 1124 cm\textsuperscript{-1}, 1396 cm\textsuperscript{-1}, 1412 cm\textsuperscript{-1} and 1694 cm\textsuperscript{-1}. These correspond to differences in chemistry between the two classes in nucleic acids (1010 cm\textsuperscript{-1}, 1040 cm\textsuperscript{-1} and 1412 cm\textsuperscript{-1}), carbohydrates (1014 cm\textsuperscript{-1}, 1030 cm\textsuperscript{-1} and 1124 cm\textsuperscript{-1}) and proteins (1396 cm\textsuperscript{-1} and 1694 cm\textsuperscript{-1}) [86, 143, 146, 152, 163].

Figure 4:24 PCA results for the 2\textsuperscript{nd} derivative data. The scores plot is shown in a) and exhibits a degree of separation. The control cell spectra are shown in red with the tet deprived cell spectra shown in blue. The loading plot for PC1 is shown in b) as the separation between the two classes is described by this PC.
A comparison of the loading plot for PC1 of the 2nd derivative transformed PCA and the mean spectra for each sample was also performed in order to determine if the previously assigned chemistry was real or the result of errors introduced during the derivatisation process (Figure 4:25). The comparison of the mean spectra with the loading plot highlights some interesting inconsistencies. The peaks in the loading plot for PC1 at 1014 cm\(^{-1}\) and 1040 cm\(^{-1}\) (Figure 4:25 highlighted in red) are the result of positive differences in the 2nd derivative mean spectra and as such may be the result of the derivatisation process. The other peak in the PC1 loading plot which has been highlighted in red on Figure 4:25 is the peak at 1694 cm\(^{-1}\). The mean spectra do not appear to differ at this point however if this region is magnified a small difference can be seen, however as the difference is so small it is possible that this may not be a difference in chemistry but the result of noise. The other differences shown in the PC1 loading plot however do correspond to differences between the mean spectra and are highlighted in yellow on Figure 4:25.

![Figure 4:25 A comparison of the mean 2nd derivative spectra for both tet control (red) and tet deprived cells (blue) with the PC1 loading plot (green).](imageurl)
4.2.1.3.2 Comparison of MC65 cells after 8 hr of tet deprivation

The mean spectra for both the control and tet deprived MC65 cells after 8 hrs of tet deprivation are shown in Figure 4:26. There appears to be a clear difference between the two classes in the amide I region with an apparent increase upon tet deprivation. There are also differences at ~1452 cm\(^{-1}\), ~1396 cm\(^{-1}\) and ~1230 cm\(^{-1}\). These correspond to differences in protein chemistry (~1452 cm\(^{-1}\) and ~1396 cm\(^{-1}\)) and nucleic acid chemistry (~1230 cm\(^{-1}\)) [86, 144, 150].

Figure 4:26 The mean spectra for both the control (red) and tet deprived (blue) MC65 cells after 8 hrs.
The separation of the two classes shown in Figure 4:27 is of a similar degree to that show by the 4 hr time point results. The loading plot for PC1 indicates the separation is due to changes in nucleic acid (~1016 cm\(^{-1}\) and 1720 cm\(^{-1}\)) and carbohydrate chemistry (~1140 cm\(^{-1}\)) \[142, 152, 162\]. The loading plot for PC1 however did not show a great deal of detail about the separation of these two classes. A PC-LDA analysis was therefore performed in an attempt to gain a more detailed picture about how these two classes differ from each other.

Figure 4:27 PCA results of the single cell spectra collected from the 8 hr time point sample. The scores plot is shown in a) and the PC1 loading plot is shown in b). The control cell spectra are shown in red with the tet deprived cell spectra shown in blue.
The results of the PC-LDA are shown in Figure 4:28. The scores plot shows a degree of separation (Figure 4:28 a)). The loading plot for DF1 is shown in Figure 4:28 b). The loading plot indicates that the main differences in chemistry between the two classes are found in the nucleic acids (~1011 cm\(^{-1}\) and ~1714 cm\(^{-1}\)), carbohydrates (~1137 cm\(^{-1}\)) and lipids (~1725 cm\(^{-1}\)) [142, 146].

**Figure 4:28** The LDA scores plot for the 8 hr tet deprivation time point are shown in a) and b) respectively. The control cell spectra are shown in red with the tet deprived cell spectra shown in blue.
4.2.1.3.3 Comparison of MC65 cells after 12 hr of tet deprivation

The comparison of the mean spectra for the control and tet deprived cell spectra after 12 hrs of tet deprivation appear to be fairly similar, although there is a slight difference between them around the amide II region (Figure 4:29). To examine the difference between the two different data sets more extensively a PCA was performed.

Figure 4:29 A comparison of the mean spectra for the control and tet deprived MC65 cell spectra after 12 hrs.
The PCA results of the 12 hr deprivation single cell experiment show some signs of separation (Figure 4:30). The separation is based on PC2 and PC5. The loading plot of PC2 indicates that changes in carbohydrate (~1030 cm\(^{-1}\) and ~1126 cm\(^{-1}\)) and lipid (~1724 cm\(^{-1}\)) chemistry are key factors in separating these two classes from each other. The loading plot for PC5 shows that bands for both nucleic acids (~1714 cm\(^{-1}\)) and lipids (~1741 cm\(^{-1}\)) are important in the separation of these two classes [142, 146, 152].

Figure 4:30 PCA results for the 12 hr time point. The scores plot is shown in a) the loading plots for both PC2 and PC5 are shown in b) and c) respectively. The control cell spectra are shown in red with the tet deprived cell spectra shown in blue.
The LDA results again show reasonable separation between the two classes (Figure 4:31). The loading plot of DF1 suggests that the separation is due to differences in carbohydrates (~1110 cm\(^{-1}\)), proteins (~1180 cm\(^{-1}\)) and nucleic acids (~1710 cm\(^{-1}\)) [146, 152].

Figure 4:31 LDA results for the 12 hr time point. The scores plot is shown in a) and the loading plot for DF1 is shown in b). The spectra for the MC65 control cells are shown in red and the tet deprived cells in blue.
4.2.1.3.4 Comparison of MC65 cells after 24 hr of tet deprivation

The mean spectra for the MC65 control and tet deprived cells after 24 hrs are shown in Figure 4:32. The mean spectra appear fairly similar over the amide I and amide II regions however there seems to be a difference between the classes over the rest of the fingerprint region. However, to determine if this difference was reflected throughout the data set a PCA was performed.

![Figure 4:32 A comparison of the mean spectra for the MC65 control and tet deprived cells after 24 hrs. The mean spectrum for the control cells is shown in red whereas the mean spectrum for the tet deprived cells is shown in blue.](image-url)
The results of the 24 hr deprivation single cell PCA show a degree of separation (Figure 4:33). This separation is based on the variance described in PC1. The loading plot for PC1 shows that the differences in variation occur at wavenumbers usually associated with nucleic acid (1716 cm\(^{-1}\)) and carbohydrate chemistry (\(\sim 1022\) cm\(^{-1}\) and \(\sim 1138\) cm\(^{-1}\)) [146, 152, 162]. In order to further investigate the separating chemistry between these two classes a PC-LDA was performed.

![Figure 4:33 PCA results for the 24 hr time point. The scores plot is shown in a) and the loading plot for PC1 in b). The spectra for the control cells are shown in red and the tet deprived cells in blue.](image-url)
The LDA results show a good separation between the two classes (Figure 4:34). The DF1 loading plot shows that this is due to changes in the chemistry of the nucleic acids (~1701 cm$^{-1}$ and ~1720 cm$^{-1}$) and carbohydrates (~1018 cm$^{-1}$, ~1036 cm$^{-1}$) between the tet deprived and the tet supplemented samples (Figure 4:34) [146, 152, 156].

Figure 4:34 LDA results for 24 hr time point. The scores plot is shown in a) with the MC65 control cell spectra depicted in red and the tet deprived cell spectra in blue. The loading plot for DF1 is shown in b).

4.2.1.3.5 Conclusion of the SR-FT-IR experiment

The results of the single cell FT-IR experiment indicate that the most important biomolecules in the separation of the tet treated and tet deprived cell samples over the first 24 hrs are the carbohydrate and nucleic acids. This would suggest that the Aβ protein had not reached detectable levels during these early time points. In order to determine if it was at all possible to locate a concentration of these Aβ proteins a third experiment was devised. This involved the use of single cell SR imaging at the
IRENI beamline. It was hoped that if a number of very high spatial resolution single cell images could be obtained from tet deprived cells after 120 hrs of tet deprivation that it may be possible to detect concentrations of Aβ proteins.

4.2.1.4 The single cell SR imaging experiment

As the previous experiments using large aperture single point FTIR and single cell SR-FTIR methodologies had largely failed to detect any accumulation of Aβ proteins within the MC65 cell upon tet deprivation, a higher spatial resolution FTIR methodology was used in a final attempt to determine if it was possible to detect these intra-cellular accumulations of Aβ protein. In order to achieve this high spatial resolution FTIR imaging using an FPA detector must be fully illuminated with SR. This is currently only available at the IRENI beamline. The MC65 cells used for this experiment were cultured for 120 hrs either in tet supplemented media as a control sample or media deprived of tet which should cause the accumulation of Aβ proteins within the MC65 cells.

4.2.1.4.1 Data acquisition

Infrared measurements were collected at the IRENI beamline at the Synchrotron Radiation Center, Wisconsin. The instrumentation setup consisted of a Bruker Vertex 70 interferometer connected to a Bruker Hyperion IR microscope equipped with a x74 objective and a x15 condenser and a FPA detector. The FPA sampling area was set to 64 x 64 pixels, with a pixel size 0.54 μm x 0.54 μm. Spectra were acquired in transmission-mode at a 4 cm⁻¹ spectral resolution with 256 co-scans for the background and sample. A total of 53 cells were imaged (29 tet deprived and 24 tet supplemented).

4.2.1.4.2 Pre-processing

Each acquired image (containing 4096 spectra) was subject to PCA-based noise reduction by retaining 30 principal components. The level of noise removal was carefully monitored by observation of the difference spectrum (noise reduced spectrum extracted from the raw spectrum). The spectra associated with the cell
were then extracted and corrected for scattering using the RMieS algorithm. Corrected spectra were then reshaped into a matrix filled with NaNs (Not-a-number) in their original positions for image recombination (Figure 4.35).

Figure 4:35 Example total intensity of absorbance infrared image of an MC65 cell before and after RMieS correction.
4.2.1.4.3 Localisation of beta component

For spatial localisation of the β-rich regions in each cell, the peak height ratio map \( \beta/(\alpha + \beta) \) was calculated for each cell \((1643 \text{ cm}^{-1} / 1643 \text{ cm}^{-1} + 1657 \text{ cm}^{-1}) \) [143, 159]. In order to assign these localisations in β-sheet to cellular architecture and therefore possible cellular processes would require the direct comparison with an optical image of the cell studied. This was however, unavailable at the time of this analysis. Each map was scaled so that the maximum value and minimum values were set to the upper adjacent value and lower adjacent values respectively (Figure 4:36).

![Figure 4:36 The scaled peak height map of (1643 cm\(^{-1}\) / 1643 cm\(^{-1}\) + 1657 cm\(^{-1}\)) highlighting β-rich regions (pink).]
4.2.1.4.4 Quality Threshold

In general, a reduction in signal is observed at the edges of each cell, which may be due to sample thickness. By thresholding-out the low-absorbing regions (based on the β/α+ β maps), we can increase the spectral quality of the average signal for each cell and also concentrate the β signal. The threshold tool creates a binary mask which is then overlaid on the original hyperspectral image to extract the desired spectra (Figure 4:37). To deduce the threshold level, a histogram of (β/α+ β) intensities is created. The cut-off point uses Otsu's method, which chooses the threshold to minimize the intraclass variance of the black and white pixels [164]. The desired spectra are then averaged to create a mean representative spectrum of each cell.

![Figure 4:37 Extracting desired spectra using the threshold tool. The white pixels (right) are the desired pixel spectra that are extracted from the cell.](image)

Particularly poor mean spectra were removed from further analysis e.g. (Figure 4:38). A total of 5 spectra from the tet deprived sample and 3 spectra from tet supplemented sample were removed, leaving 24 minus and 21 plus representative cell spectra.
Figure 4:38 Bad spectra removed from further analysis: tet deprived cells 1, 2, 3, 4 and 21 as well as tet supplemented cells 3, 5 and 15. Mean spectra for each sample is shown in green with the standard deviation (std) either added (blue) or subtracted (red).
4.2.1.4.5   Analysis of the mean fingerprint spectra

Figure 4:39 displays the mean fingerprint spectra for the tet deprived and tet supplemented cells that were used for analysis. The tet supplemented cell spectra are shown in red and the tet deprived cell spectra in blue. There seems to be large variation in the absorbance values over the whole data set. At this stage, however, no normalisation has been performed.

![Absorbance vs Wavenumber graph](image)

Figure 4:39 The mean spectra for each cell imaged. The tet supplemented cell spectra are shown in red whereas the tet deprived cell spectra are shown in blue.
After normalisation, differences in thickness are reduced (Figure 4:40). This may reduce cell-cycle chemical variance, however, as dividing cells will be thicker. It is also clear from this figure that two of the tet deprived spectra are very different from the rest of the data set and as such were removed from further analysis.

Figure 4:40 The mean spectra after vector normalisation. The tet deprived cell spectra are shown in blue and the tet supplement cell spectra in red.
The cell spectra were grouped together; vector normalised and combined to illustrate the mean spectra for both the tet deprived sample and the tet supplemented sample. Figure 4:41 displays the mean fingerprint spectra and standard deviation for both the tet deprived and tet supplemented classes.

![Graph showing mean fingerprint spectrum for tet deprived and tet supplemented samples.](image)

**Figure 4:41** Mean fingerprint spectrum for the tet deprived sample (blue) and tet supplemented sample (red) ± std deviation. A slight offset was applied to the tet supplemented spectrum for clarity.

There appears to be no obvious difference in the amide I shoulder of each group. In the second derivative, however, there does appear to be differences in the β-associated protein regions at 1694 cm\(^{-1}\) and 1643 cm\(^{-1}\). There is a reduction in peak height in both cases of anti-parallel and parallel β sheet contribution in the tet supplemented class (Figure 4:42).
Figure 4: 2\textsuperscript{nd} derivative mean spectra for the tet deprived (blue) and tet supplemented (red) classes. The antiparallel and parallel β-sheet associated peak positions at \( \sim 1694 \text{ cm}^{-1} \) and \( \sim 1643 \text{ cm}^{-1} \) are labelled a) and b) respectively, either side of the α-helix-associated position at \( \sim 1657 \text{ cm}^{-1} \) [143, 159].

4.2.1.4.6 Conclusion of the FT-IR imaging experiment

The results of the FT-IR imaging are consistent with those of the large aperture single point experiment in that upon 2\textsuperscript{nd} derivatisation it is possible to detect an increase in the intracellular levels of β-sheet associated vibrations. However disappointingly these were still not evident in the underivatised spectra and as such this cell line was not considered further for use with the microfluidic device. In order to determine why the levels of Aβ did not reach the expected levels of detection some investigation into the amount of Aβ secreted should be performed to determine whether or not these cells were producing the quantity of Aβ expected. Another interesting experiment would be to attempt to purify and analyse the total protein extract from a sample of tet deprived cells and see if an increase in β-sheet vibration could be detected using FT-IR. Unfortunately due to time limitations of this project, these further experiments could not be performed.
4.2.2 Conclusion of the MC65 cell line assessment

The results of the three MC65 cell line experiments are unfortunately not very conclusive. The data analysis suggests that it may be possible to detect a slight increase in the Aβ sheet content of the MC65 cells after 120 hrs. This is not however the gradual increase in β-sheet vibrations over time which was hoped for and which would be necessary for the testing of the water removal algorithm. It was therefore concluded that this cell line was not suitable for use in the development of the microfluidic device. The next candidate cell lines for use with the microfluidic device were the suspension cells K562 and Nalm1.

4.3 Suspension cells

As the aim of this project was to develop a device in which cells could be flowed in to the analysis chamber, a FT-IR spectrum collected and the cells flowed out again. A disadvantage of the previously discussed cell lines was that they possessed an adherent phenotype. This means that these cell types require attachments to a solid surface in order to proliferate [165]. The prevention of proliferation can cause significant changes in cell biochemistry as the cells will pass into senescence (a state of hibernation) and eventually die [166]. For this reason a better cell type for the initial testing and development for the device would be a suspension cell type. Suspension cells are also referred to as non-adherent cells as they do not require an attachment to a solid surface in order to proliferate. Suspension cell lines are usually descended from blood cells [165]. By choosing cell lines specifically from blood lineages the cells should also be more resistant to mechanical stress such as that encountered within this device. This is due to the fact that these cell types are designed to be present in the circulating blood and in the case of T cells and Monocytes, to pass out of small spaces in the capillary walls and in to the surrounding tissue [4].
The suspension cell lines which have been chosen for use in this project are the Nalm1 and K562 cell lines. The Nalm1 cell line is described as a pre B cell whereas the K562 is a myelogenous cell line [167-168]. In order to fully understand what these cell classifications mean and how the two cell lines are related the differentiation pathway of hemopoietic cells is shown in Figure 4:43.

As can be seen in Figure 4:43 both the K562 (common myeloid progenitor) and Nalm1 (Pre B cell) cell lines, can be thought of as models of committed progenitor cells. These are cells that are not fully differentiated and may even be able to differentiate into several different cell types; however they have lost the ability to differentiate in to any cell type within the tissue and so can no longer be classed as a stem cell. One important difference to note between the K562 and Nalm1 cell lines is that the K562 cell line is still capable of differentiating in to several different cell types whereas the Nalm1 cell line can only become a fully differentiated B-cell. The K562 cell line should therefore have a less differentiated biochemistry than the Nalm1 cell line. A second difference between the two cell lines is the role of the cell types that they finally differentiate in to. The Nalm1 terminal cell is a member of the...
specific (adaptive) immune system whereas the myeloid progenitor differentiates in to members of the non-specific (innate) immune system [170].

Due to the differences between these two closely related cell lines it should be possible to distinguish between them using live cell FT-IR analysis. Although previous studies have been done using FT-IR and fixed K562 cells, no previous work has been performed using the Nalm1 cell line with FT-IR [171-172]. Therefore a fixed cell study was needed to determine if it was possible to separate the FT-IR spectra of Nalm1 cells from those of the K562 cells on CaF₂ substrates. In order to achieve this a combination of FT-IR and chemometric techniques such as those used to differentiate between other cell lines by groups such as Hughes et al. and Harvey et al. were used [29, 173]. Once it had been established that discrimination was possible between these cell lines using this combination of techniques it would be possible to attempt analysis of FT-IR spectra obtained from fixed cells in solution using the microfluidic device. Provided that this also proved possible, moving on to live cell analysis would be achievable.

4.3.1 Methodology

4.3.1.1 Cell culture

The K562 cell line was cultured in RPMI 1640 media with 10% foetal bovine serum (FBS), 2mM L-Glutamine (Gln) and 2% Penicillin and Streptomycin (Pen\Strep). The cell line was maintained at a cell density between 1x10⁵ and 1x10⁶ cells/ml at a constant temperature of 37.5 °C and 5% CO₂ as per European Collection of Cell Cultures (ECACC) guidelines [174]. The Nalm1 cell line was cultured in RPMI 1640 media with 15% FBS, 2mM Gln and 2% Pen\Strep. The cell line was maintained at a cell density of between 1x10⁶ and 2x10⁶ cells/ml at a constant temperature of 37.5 °C and 5% CO₂ as per DSMZ guidelines [175].

4.3.1.2 Sample preparation

A 1ml aliquot of 1x10⁶ cell suspension was obtained from the culture flasks and centrifuged at 1200 rpm in a microfuge (eppendorf minispin). The supernatant was then removed and the cells resuspended in PBS (PAA). The purpose of this wash
step was to remove traces of the culture media from the cell sample which may affect the analysis. The cells were then centrifuged and the supernatant removed as previously described 4.2.1.1.2. The cells were then resuspended in a 4% formalin in PBS solution (2ml) for fixation for a minimum of 20 mins after as per Gazi et al. [31]. The fixed cell suspension was then centrifuged as before and the supernatant removed and replaced with PBS. A 100 μL aliquot of this cell suspension was then removed and used for cell deposition on to CaF$_2$ substrates by cytocentrifugation at 1200 rpm (Thermo Scientific Cytospin). The CaF$_2$ substrates were then left to air dry overnight after which they were dipped in distilled water 3 times for a period of 3 s to remove any crystallised PBS from the sample surface. The samples were then left to air dry before being stored in a desiccator (Secador) prior to FT-IR analysis.

4.3.2 FT-IR of Nalm1 and K562 cells

4.3.2.1 SR-FT-IR

The single cell synchrotron data for both cell lines was obtained at the B22 beamline based at the Diamond light source located near Didcot in the UK. The data was collected in transmission mode using a 36x objective with an aperture size of 15 μm x 15 μm at 4 cm$^{-1}$ resolution and 256 co-scans, on the Bruker Vertex 80v Vacuum-FT-IR with Hyperion 3000 IR microscope (Endstation 2).

4.3.2.2 FT-IR spectral imaging

The imaging data was collected on a Varian 620-IR FT-IR spectrometer coupled to a Varian 620-IR FT-IR microscope with a 128 x 128 FPA available in house. The imaging data was also collected in transmission mode however a 15x objective was used as opposed to the 36x at diamond. The same spectral resolution was used as at Diamond however, the number of co-scans was increased to 512 as opposed to 256.
4.3.3 Single cell SR-FT-IR results

The mean spectra for both the K562 and Nalm1 cell samples are shown in Figure 4:44. The mean spectrum for the Nalm1 cell sample is shown in blue and the K562 mean spectrum in red. The main differences between the mean spectra appear to be in the amide II and protein associated methyl region ~1455 cm\(^{-1}\) [143-144]. There also appears to be a difference at ~1080 cm\(^{-1}\) and 1170 cm\(^{-1}\) these are usually associated with nucleic acids and glycomaterials respectively [146-147]. There also appears to be a significant difference in the lipid region (~2800 cm\(^{-1}\) – ~3200 cm\(^{-1}\)) between the two classes. In order to further examine the differences between these two cell types a PCA was performed.

![Figure 4:44 A comparison of the mean spectra for the Nalm1 (blue) and K562 (red) cell samples.](image)
The PCA was performed on the ‘fingerprint’ region between 960 cm\(^{-1}\) and 1750 cm\(^{-1}\) and the lipid region between 2800 cm\(^{-1}\) and 3200 cm\(^{-1}\) separately. This was done because the spectral region between these two analysis substrates is devoid of any biologically relevant spectral information. The results for the fingerprint region of this data set are shown in Figure 4:45 a). There is good separation between the two cell lines when the data is plotted using PC2 against PC3. The loading plots for PC2 and PC3 are also shown in Figure 4:45 b) and c) respectively.

![Figure 4:45 a)](image1)

**Figure 4:45 a)** depicts a scores plot for the single cell data obtained on the B22 beamline at the diamond synchrotron. The K562 cells are pictured in red with the Nalm1 cells in blue. The loading plots of both PC2 and PC3 are also shown in b) and c) respectively.

The separation in PC2 is mainly based on the peaks at \(\sim 1175\) cm\(^{-1}\), \(\sim 1340\) cm\(^{-1}\) and \(\sim 1485\) cm\(^{-1}\). The absorbance band at \(\sim 1175\) cm\(^{-1}\) equates to C-O stretching vibrations usually associated with proteins or carbohydrates. The absorbance band at \(\sim 1485\) cm\(^{-1}\) corresponds to the amide II which is also a protein band. The absorbance band at \(\sim 1340\) cm\(^{-1}\) is reported to be associated with the CH\(_2\) wagging vibration [86, 143, 151-152].
The separation shown in PC3 however is slightly different with bands occurring at ~1055 cm\(^{-1}\), ~1107 cm\(^{-1}\) and ~1705 cm\(^{-1}\). The bands at ~1055 cm\(^{-1}\) and ~1107 cm\(^{-1}\) are both polysaccharide vibrations. The 1705 cm\(^{-1}\) band however is a nucleic acid vibration [142, 152].

PC-LDA was used to further investigate the separation between these two classes and to determine which differences in chemistry between them contributed the most to the class separation. The LDA scores plot is shown in Figure 4:46 a), the Nalm1 cell spectra are coloured in blue and the K562 in red. The scores plot indicates a degree of separation between these two cell types over the fingerprint region.

![Figure 4:46 a) Score and b) loading plots for the LDA of the fingerprint region. Nalm1 cells are shown in blue while the red dots correspond to the K562 cell spectra.](image)

The DF1 loading plot in Figure 4:46 b) shows that the separation between the two classes in the LDA is mainly based on three main peaks at ~1126 cm\(^{-1}\), ~1697 cm\(^{-1}\), and 1712 cm\(^{-1}\). There are however, also several other peaks which also influence the separation at ~1041 cm\(^{-1}\), ~1053 cm\(^{-1}\), ~1172 cm\(^{-1}\), ~1205 cm\(^{-1}\), ~1307 cm\(^{-1}\),
~1348 cm\(^{-1}\), ~1369 cm\(^{-1}\) and ~1496 cm\(^{-1}\). These bands stem from changes in nucleic acids (1041 cm\(^{-1}\), 1369 cm\(^{-1}\), 1697 cm\(^{-1}\), 1712 cm\(^{-1}\)), carbohydrates (1053 cm\(^{-1}\), 1126 cm\(^{-1}\), 1205 cm\(^{-1}\)), proteins (1172 cm\(^{-1}\), 1307 cm\(^{-1}\), 1348 cm\(^{-1}\)) and lipids (1496 cm\(^{-1}\)) [145-146, 149-152, 157].

The results of the PCA for the lipid region are presented in Figure 4:47. The results show some separation based on PC1. In the loading plot for PC1, although several distinct bands can be seen these all correspond either C-H vibrations or the amide B. The lipid vibrations found in the PC1 loading plot are not specific to either unsaturated or saturated lipid molecules but common to both, and as such do not add anything further to the biochemical changes shown in the fingerprint analysis. The same is true of the amide B protein vibration which the change in, although corresponding to a difference in protein chemistry, does not add any further detail to this difference in biochemistry already shown in the fingerprint region.

Figure 4:47 PCA results for the lipid region analysis comparing the single spectra of Nalm1 and K562 formalin fixed cells. Nalm1 cells are shown in blue while the red dots correspond to the K562 cell spectra.
4.3.4 FT-IR imaging results

The first step in the analysis of the imaging data was to extract the single cell spectra from the raw image. Some examples of the raw FT-IR images for the Nalm1 and K562 cell lines are shown in Figure 4:48.

![Raw FT-IR total absorbance images for the K562 (a) and b) and Nalm1 (c) and d) cell lines.](image)

Using these raw total absorbance images as a base it is possible to create a binary image displaying the position of each individual cell (Figure 4:49 a)). Using the binary image it is then possible to determine the boundary of each cell and assign the pixels to the appropriate cell. The mean of the pixels corresponding to each cell can then be calculated. This mean is then used as the single cell spectrum in all further analysis. One problem with this method is that if the cells are too close together the boundary between the individual cells cannot be determined and cells
are averaged together. The cells labelled 27, 28, 65 and 100 and outlined in red in Figure 4:49 b) are some examples of when this has occurred.

![Image: Figure 4:49 a) shows the binary mask for one of the Nalm1 FT-IR images and b) shows the individual cell boundaries indicating which pixels are averaged to create each cell spectrum.]

The comparison of the mean spectra for both data sets suggests that there is a difference in chemistry between the cell lines in both the fingerprint (~1000 cm\(^{-1}\) - ~1800 cm\(^{-1}\)) and lipid regions (~2800 cm\(^{-1}\) – 3200 cm\(^{-1}\)). In order to effectively study these differences the fingerprint and lipid regions were analysed separately using PCA.

![Image: Figure 4:50 A comparison of the mean spectra for the K562 and Nalm1 cell lines. The Nalm1 mean spectrum is shown in blue and the K652 mean spectrum in red.]
The PCA results for the fingerprint region of the FT-IR imaging study are presented in Figure 4:51. The scores plot clearly shows that there are chemical differences between the two cell lines and that these can be detected by FT-IR (Figure 4:51 a)). The loading plots for PC1 and PC2 which together define this separation are shown in Figure 4:51 b) and Figure 4:51 c) respectively. PC1 indicates that there are differences in the carbohydrate chemistry (~1050 cm$^{-1}$, ~1110 cm$^{-1}$ and ~1170 cm$^{-1}$), the protein chemistry (~1196 cm$^{-1}$ and ~1350 cm$^{-1}$) and in the nucleic acids present (~1705 cm$^{-1}$). PC2 also shows differences in carbohydrate chemistry (~1053 cm$^{-1}$ and 1427 cm$^{-1}$), however there is a much greater influence on the separation from the nucleic acids (~1041 cm$^{-1}$, 1115 cm$^{-1}$, 1354 cm$^{-1}$ and 1701 cm$^{-1}$) and proteins (1173 cm$^{-1}$, 1196 cm$^{-1}$ and 1481 cm$^{-1}$) in this PC [143, 145, 152, 157, 176].

Figure 4:51 PCA results for the imaging data comparing the Nalm1 and K562 cell lines. The scores plot is shown in a) with the loading plots for PC1 and PC2 in b) and c) respectively. The Nalm1 cell spectra are shown in blue and the K562 cell spectra in red.
As the separation of the PCA is best described by more than one PC, PC-LDA was performed in order to determine exactly which chemistry was most important in the separation. The PC-LDA results are presented in Figure 4:52. The separation between the two classes shown in the scores plot is reasonable (Figure 4:52 a)). The DF1 loading plot shows that the differences in the two cell lines stem from variation in the nucleic acid (~1045 cm\(^{-1}\), ~1080 cm\(^{-1}\) and ~1705 cm\(^{-1}\)) and carbohydrate (~1110 cm\(^{-1}\) and ~1153 cm\(^{-1}\)) chemistry of the cells (Figure 4:52 b)) [86, 146, 152]. This is consistent with the single cell data analysis which also concluded that the differences in carbohydrate and nucleic acid chemistry were mainly responsible for the separation of the two cell lines.

Figure 4:52 LDA results; the scores plot is shown in a) and the loading plot for DF1 is depicted in b). The Nalm1 cell spectra are shown in blue and the K562 in red.
The lipid analysis of the imaging data was also performed. The PCA results are depicted in Figure 4:53. The class separation of the PCA is not as well defined as that of the fingerprint region. The loading plot for PC2 which describes the separation between the two classes does not show any difference in the unsaturated lipid chemistry which is the main reason for using this spectral region. This fact combine with the relatively poor class separation suggests that any further experimental analysis should be limited to the fingerprint region.

Figure 4:53 PCA for the lipid region of the K562 and Nalm1 comparison. The scores plot is shown in a) with the loading plots for PC1 and PC2 in b) and c) respectively. The Nalm1 cell spectra are shown in blue and the K562 cell spectra in red.
4.3.5 Conclusion of the suspension cell experiments

The results of the imaging and SR FT-IR analysis of the fixed Nalm1 and K562 cells on CaF$_2$ substrates showed that it was possible to differentiate between these two closely related leukaemic cell lines using both of these FT-IR methodologies in combination with multivariate analysis. The results also showed that the greatest separation between the two cell lines was in the fingerprint region of the spectrum and therefore only this spectral region was considered during the evaluation of the microfluidic device.
CHAPTER 5  Development of the buffer removal program

The cell spectra obtained from cells in an aqueous solution are very different to those obtained from cells which have been prepared for analysis by traditional means. An example of a cell spectrum obtained in PBS buffer solution at the diamond synchrotron and that of a cell of the same cell line cytospun on to a CaF$_2$ substrate and measured using an FT-IR imaging system are shown in Figure 5:1 a) and b) respectively.

![Figure 5:1](a) single cell spectrum of a cell in aqueous solution, b) the spectrum of a single cell after RMieS correction obtained by FT-IR imaging and c) example PBS buffer spectrum.

It is immediately clear from Figure 5:1 that the spectrum of the cell in buffer solution bears a much greater resemblance to that of the buffer spectrum as opposed to that of the RMieS corrected spectrum. It is for this reason that the buffer contribution to any cell spectral data obtained in buffer solution needs to have the buffer contribution removed prior to multivariate analysis.

The easiest method of doing this is to perform a spectral subtraction. This however is not as simple as it sounds as there are many ways of doing this. The most commonly used methods are those available in the commercially available IR software such as OPUS and OMNIC. These programs are all similar in that they
require the user to ‘fine tune’ the subtraction by use of a sliding scale until the spectral product look about right. Screen shots of the OMNIC and OPUS spectral subtraction windows are presented in Figure 5:2 a) and b).

The main disadvantages to this method is that the fitting of the buffer spectrum to the obtained spectrum (hence forth referred to as raw spectrum) is subjective and may therefore result in inaccuracies in the analysis of the data. It is also very time
consuming since each spectrum has to be background subtracted manually. A second method of spectral subtraction has been employed by Birarda et al. 2010. Birarda and co-workers used a java routine to fit the buffer and raw spectrum. The fitting was based on the water combination band at 2100 cm\(^{-1}\) [52]. This is an improvement on the commercial software as the subjective element of the fitting routine has been removed. The fitting method that was used for the buffer removal in this project however was different to both of these procedures.

5.1 The buffer removal program

The aim of the buffer removal algorithm is to convert the spectrum of the cell obtained in aqueous media to one of just the cell in its natural aqueous environment but without the confounding water spectrum. This recovered cell spectrum should resemble (to some degree) the spectrum of a cell in a fixed dried state. The spectra will not be a perfect match since the fixation process will induce some changes, for example dehydration of DNA molecules can cause a change in their conformation and cross linking of proteins may affect the spectral signatures detected [48]. A graphical representation of the water removal process is shown in Figure 5:3.
The most important part of this process is how the fitting of the buffer spectrum to the raw spectrum is performed. The fitting algorithm used for the buffer removal in this project was the least squares fitting algorithm.

The Least squares fitting method aims to fit data such that the residual of Equation 5-1 in is minimised [128].

**Equation 5-1** \[ \text{Residual} = (RP - M \times R)' \times (RP - M \times R) \]

Where \( RP \) are a set of coefficients which represent the regression parameters, \( M \) is a matrix of the continuants that are thought to be in \( R \). \( R \) is a matrix containing the sample data.
This can be used as a fitting method to facilitate the buffer removal by assigning RP, M and R as in Equation 5-2 [128].

\[
\begin{align*}
R & = \text{WN} \\
R & = \text{WN} \\
\end{align*}
\]

\[
\begin{align*}
\text{Equation 5-2} \\
R & = \text{WN} \\
R & = \text{WN} \\
\end{align*}
\]

Where R is the sample spectrum, RP are the regression parameters which equate to the proportion of each constituent (c) in the model (M) which are present in the sample spectrum. WN represents the wavenumber variable.

The model variable described in equation 4-2 is a matrix composed of a matrigel reference spectrum and a buffer spectrum. The matrigel reference spectrum is used in the model in order to preserve the amide I band of the cell spectrum which may otherwise be overly subtracted. The correct amount of buffer spectrum to be subtracted from the raw sample data is therefore determined by the multiplication of the buffer spectrum by the RP for the buffer spectrum as previously illustrated in Figure 5:3. It should also be noted that a weighting variable is used to prevent the inclusion of the CO₂ and H₂O stretching regions in the buffer fitting process.
5.2 Validation of the buffer removal algorithm

The initial testing of the buffer removal algorithm was performed on spectral models of aqueous cell spectra. These spectral models were created by combining an ideal water spectrum to represent the buffer solution and a spectrum of a thin film of matrigel. The matrigel film is free of scattering artefacts and is representative of a Generic biological spectrum. This has been used very successfully to correct cell spectra contaminated by scattering so it is reasonable to use it here [34, 118, 122, 177]. The starting point for the model spectrum was a ratio of 1:0.01 water to matrigel, this ratio was chosen as the spectral product appeared to be similar to the aqueous cell spectra obtained from the microfluidic device. The results of the buffer removal process are shown in Figure 5:4.

![Graphical evaluation of the buffer removal program](image)

Figure 5:4 graphical evaluation of the buffer removal program. a) is the artificial aqueous cell spectrum, b) is a comparison of the matrigel spectrum compare to the result of the buffer removal program, c) is a plot of the difference between the two spectra across the wavenumber range and d) is the percentage error across the same wavenumber range.

The results in Figure 5:4 were very positive with differences between the two spectra in the order of $10^{-16}$ and a maximum percentage error of $5 \times 10^{-10}$. The cellular content of the initial model was only $1 \times 10^{-2}$, so based on this modelling of the performance any errors introduced in to the buffer removed spectrum from the buffer removal process would be in the order of $1 \times 10^{-8}$ well below a reasonable level of noise. This model is only a measure of the buffer removal programs ability to
remove buffer contributions under perfect conditions i.e. a perfect reference for both buffer and cellular content, as well as there being no noise contributions from atmospheric differences. A second evaluation of the buffer removal spectrum was therefore performed. This test was to involve the use of both actual cell spectra and a actual buffer solution spectrum; however the buffer was still added to the cell spectra as a known quantity in the same ratio as before. The buffer spectrum used was a PBS spectrum obtained in the microfluidic device using SR-FT-IR. The cell data was a single point open aperture spectra of four different prostate cancer related cell lines. The cell lines used were the PC3, PNT2, LNCaP and BPH. The PC3 cells are grade IV epithelial prostate adenocarcinoma cells that were isolated from a metastatic site in the patient's bone [178]. The PNT2 cells are normal prostate cells which have been immortalised by inserting a plasmid containing the SV40 genome however, the plasmid is unable to copy itself as the replication origin has been modified [179]. The LNCaP cells are epithelial-like prostate adenocarcinoma cells that were isolated from a metastasis in a lymph node [179]. The BPH cells were obtained from primary tissue. A single spectral example of the before and after buffer removal as well as a comparison of the two spectra can be found in Figure 5:5.
Figure 5:5 a) example spectrum of cell spectrum before addition of the buffer spectrum, b) example spectrum with the addition of the buffer spectrum, c) spectrum after buffer removal and d) comparison of the cell spectrum before and after buffer removal spectra.

The results of PCA of both the data prior to buffer removal and after buffer removal are shown in Figure 5:6. Although there is a slight difference between the two scores plots the basic separation between the classes is very similar. The comparison of the PC loading plots also shows good correlation between the before and after buffer removal.
Figure 5:6 a) PCA of the data set prior to buffer addition, b) PCA of data after buffer removal, c) comparison of PC1 loadings before and after buffer removal and d) comparison of PC3 loadings before and after buffer removal. The loading data prior to buffer addition is shown in blue whereas the loadings in green represent the buffer removed data.
5.3 Conclusion

The testing of the buffer removal program shown in this chapter demonstrates that this least squares based fitting algorithm is able to remove the buffer contribution from a cell spectrum under theoretical conditions. Although there are some slight differences in the loading plots shown in Figure 5:6 these slight variations were not significant enough to affect the classification of the spectra. The buffer removal program was therefore considered ready for use in the analysis of actual aqueous cell spectra such as that shown in CHAPTER 6 and CHAPTER 7.
CHAPTER 6 Results of FT-IR microfluidic device experiments

The initial experiments conducted using the microfluidic device centred on repeating the results obtained for the fixed suspension cell data previously discussed in 4.3 and comparing the data obtained from both experiments to further evaluate the water removal algorithm previously discussed in CHAPTER 5. As the analysis of the fixed cells on CaF$_2$ substrates found that the most informative chemical differences between the two cell lines was found in the fingerprint region (~1000 cm$^{-1}$ - ~1800 cm$^{-1}$) the analysis window for this experiment was limited to this region. However this had to be further limited to between ~1400 cm$^{-1}$ and 1750 cm$^{-1}$ due to the use of two 1 mm CaF$_2$ substrates in the construction of the microfluidic device and the effect this has on the low wavenumber cut-off.

6.1 Fixed cell analysis of K562 and Nalm1 cell lines

The spectra were obtained from formalin fixed Nalm1 and K562 cells which were cultured and formalin fixed according to the methods previously described in 4.3.1.1 and 4.3.1.2 respectively. The cell suspension medium was changed from 4% formalin solution to PBS prior to being run through the microfluidic device for analysis to minimise any signal from the carrier fluid.

The single cell FT-IR spectra were collected at the Diamond Light Source synchrotron facility. The spectra were obtained at 4 cm$^{-1}$ resolution using 256 co-scans. The aperture size used was 15 μm x 15 μm. Each individual cell spectrum was collected with a matched pair buffer spectrum from a cell free region of the device, as near to the cell as possible.

The spectral data was pre-processed slightly differently to the standard fixed cell data in that the spectra had to have the buffer contributions removed prior to data analysis. This was done using the water removal algorithm previously discussed (see CHAPTER 5), the spectra were also vector normalised. However, due to the refractive index matching of the buffer solution to the CaF$_2$ substrates used in the construction of the analysis chamber the spectra did not suffer from noticeable Mie scattering and as such the use of the RMieS algorithm was unnecessary.
6.1.1 Results of the fixed cell analysis of the K562 and Nalm1 cell lines

The mean spectra for the formalin fixed Nalm1 and K562 samples after buffer subtraction has been performed and are shown in Figure 6:1. There appears to be a slight off-set in the baseline between the two samples however to determine if this problem was present throughout the data set a PCA was done.

![Figure 6:1 A comparison of the mean spectra for the aqueous formalin fixed Nalm1 (blue) and K562 (red) cell samples.](image)
The results of the PCA analysis are presented in Figure 6:2. The scores plot shows separation between the K562 and Nalm1 cell spectra along the PC1 axis (Figure 6:2 a)). The loading plot for PC1 does appear to show some changes in chemistry however the fact that the two extremes of the loading plot are the source of the greatest separation could indicate a contribution to the separation from a change in the baseline between the two groups. As this is the conclusion from both the mean spectral analysis and the PCA, the spectra were transformed to the second derivative in order to eliminate any base line contributions.

Figure 6:2 PCA of the single fixed cell spectra obtained in PBS solution using the microfluidic device. Cell spectra were collected for both K562 and Nalm1 cells. The PCA scores and loadings for PC1 are plotted in a) and b) respectively.
The PCA results for the second derivative spectra are shown in Figure 6:3. The scores plot shows clear separation between the two groups along the PC2 axis (Figure 6:3 a)). The loading plot for PC2 suggests that the major biochemical difference between these 2 cell lines is in the protein chemistry (~1655 cm\(^{-1}\) and ~1550 cm\(^{-1}\)) (Figure 6:3b)) [24, 142].

Figure 6:3 PCA of the second derivative spectra. The scores plot and the loading plot for PC2 are shown in a) and b) respectively. Nalm1 cell spectra are shown in blue and K562 cell spectra in red.
Although the two bands previously mentioned offer the greatest contribution to the separation of the K562 and Nalm1 cell spectra collected in this experiment, the other smaller bands may also offer interesting information about the separation. It is however easy to confuse these smaller changes with noise. To avoid this, a comparison between the loading plot for PC2 and the mean spectrum for both K562 and Nalm1 cell lines was performed, the result of which is shown in Figure 6:4. The smaller changes which correspond to visible differences between the mean spectra for the K562 and Nalm1 cell lines have been highlighted in yellow. These differences can be found at ~1633 cm\(^{-1}\), ~1469 cm\(^{-1}\) as well as at ~1454 cm\(^{-1}\). These band positions correspond to variations in the protein chemistry specifically in the β-sheet secondary structure (~1633 cm\(^{-1}\)), as well as differences in the lipid chemistry (~1469 cm\(^{-1}\) and 1454 cm\(^{-1}\)) [86, 143, 162].

Figure 6:4 A comparison of the loading plot for PC2 (green) and the mean cell spectrum for K562 (red) and Nalm1 (blue). The major differences in chemistry are highlighted in yellow.

### 6.2 Live cell analysis of the Nalm1 and K562 cell lines

Prior to collecting any live cell spectra it was necessary to assess the effect of the PBS carrier fluid and nutrient deprivation on the cell viability. In order to study this, an aliquot of cell PBS solution was monitored over a number of time periods. A sample of cells was removed from the PBS cell solution at regular intervals and the cell viability was measure by trypan blue dye exclusion. The results of this experiment are presented in Figure 6:5. The results for both cell lines indicate that
there is no significant drop in cell viability until the cells have been suspended in PBS alone for 6 hrs. During the live cell microfluidic investigation detailed in this chapter the live cell aliquots were suspended in PBS and analysed within 1 to 2 hrs.

Figure 6:5 The viability of K562 (blue) and Nalm1 (red) cells suspended in PBS over varying time periods.
The single live cell spectra were collected and pre-processed as previously stated in 6.1. The mean spectra for the Nalm1 and K562 cell lines are shown in Figure 6.6. There also appears to be a baseline off-set between the two samples here however a PCA was performed to determine if this was the case throughout the data set.

Figure 6.6 A comparison of the mean spectra for the Nalm1 (blue) and K562 (red) cell lines.
The PCA results for this experiment are shown in Figure 6:7. The PCA scores plot clearly shows separation between the two classes in PC1 (Figure 6:7 a)). The separation described in the PC1 loading plot however suggests that a baseline offset is at least partially responsible for this separation (Figure 6:7 b)). In order to remove the contributions of this baseline effect from the analysis a second derivative transform was performed on the spectra and the analysis repeated.

![Figure 6:7 PCA results for the comparison of single live cell synchrotron spectra for the Nalm1 and K562 cell lines. The scores and loading plots of the PCA are shown in a) and b) respectively. The Nalm1 cell spectra are shown in blue and the K562 spectra in red.](image)
The analysis for the second derivative spectra is shown in Figure 6.8. The first point to notice is that the separation in the scores plot is much improved. The loading plot for PC1 is also now much more informative with the peaks responsible for the separation much more clearly defined. The biochemical differences described in PC1 are mainly differences in the protein vibrations (~1653 cm⁻¹ and ~1550 cm⁻¹) [24, 143].

Figure 6.8: PCA of the second derivative data. The scores and PC1 loading plots are shown in a) and b) respectively. The Nalm1 cell spectra are shown in blue and the K562 spectra in red.
There are however several other bands in the PC1 loading plot which appear to contribute to the separation of the two classes. In order to determine if it was possible to gain any further information from the loading plots a comparison between the loadings and the mean spectrum of each class was performed. The result of this is shown in Figure 6:9. As can clearly be seen some of the smaller peaks in the loading plot correspond to visible differences between the mean spectra. The positions of these bands as well as the previously mentioned protein bands at ~1655 cm\(^{-1}\) and 1550 cm\(^{-1}\) have been highlighted by placing a box around the corresponding spectral features in all 3 spectra (Figure 6:9). These extra bands occur at ~1635 cm\(^{-1}\) (protein β-sheet vibration), ~1510 cm\(^{-1}\) (lipids), ~1468 cm\(^{-1}\) (lipids) and ~1452 cm\(^{-1}\) (lipids) [143, 145, 152].

![Figure 6:9 comparison of the PC1 loading (red) and the mean spectrum of both Nalm1 (blue) and K562 (green) cell spectra. The mean spectra have been transformed to the second derivative in order to be comparable to the loading plot.](image-url)
6.3 Comparison of the fixed and live cell experiments for K562 and NALM1 cell lines

In order to properly compare the results for the fixed cells on CaF$_2$ substrates with that of the data obtained in the microfluidic device the wavenumber range for both data sets must be the same. To achieve this, the analysis for the CaF$_2$ substrate work was repeated over the smaller wavenumber range used for the microfluidic device work. The data collected from the fixed cells on CaF$_2$ substrates also had to be transformed to the second derivative as all the data had to be processed in as similar way as possible in order for a good comparison and therefore a good evaluation of the buffer removal algorithm to be performed.

The results for the loading plot comparisons are shown in Figure 6:10. The plots for the live (Figure 6:10 b)) and fixed (Figure 6:10 d)) cell analysis of the microfluidic data show very good correlation and also similar differences in biochemistry to that of the fixed cell imaging data (Figure 6:10 a)). The fixed single cell CaF$_2$ substrate data (Figure 6:10 c)) however appears to show a large difference from the other plots in the amide I region. This may well be due to poor signal to noise due to variations in the atmospheric water vapour signal masking the changes in this region. The changes in the lipid chemistry however, appear to be fairly consistent between all 4 of the data sets.
Figure 6:10 The mean cell spectrum for Nalm1 (blue) and K562 (red) cell lines, as well as the loading plot for the most discriminating PC or DF (green) are shown for each of the data collection methods. a) data collected by SR-FT-IR b) live cell spectra c) fixed single cell collected by FT-IR imaging and d) fixed cells in PBS.
In order to further validate the buffer removal algorithm a comparison between the fixed single-cell data collected using the FTIR imaging system and the fixed single-cell before and after buffer removal for both the K562 and Nalm1 data sets was performed. In order to compare the three classes PCA was carried out. The scores plots for both the K562 and Nalm1 data sets are shown in Figure 6:11 a) and b) respectively. The fact that the aqueous cell spectra after the buffer contribution has been removed (red) cluster closer, to the fixed cell spectra obtained using FTIR imaging (green: ~60 spectra), than the aqueous cell spectra prior to buffer removal (blue), indicates that the buffer removal algorithm does work. The consistency of the placement of the three classes in both cell lines is also interesting. The loading plot for PC1 (Figure 6:11 b) and e)) in both cases strongly resembles a buffer spectrum with the fixed single cell imaging and single aqueous cell spectra after buffer removal exhibiting a much lower level of this PC than the aqueous cell spectra prior to buffer removal. The loading plot for PC2 (Figure 6:11 c) and f)) in both cases also hints at possible spectral differences between the single fixed cell imaging spectra and the single aqueous cell spectra after buffer removal. These differences could be due to the difference in hydration state between these two classes.
Figure 6:11 The scores plots for the K562 and Nalm1 data sets are shown in a) and d) respectively. In each case the fixed single cell spectra obtained using FTIR imaging are shown in green, the aqueous single fixed cell spectra prior to buffer removal are shown in blue and the same cell after buffer removal are shown in red. The loading plots for PC1 (b) and e)) and PC2 (c) and f)) for each PCA are also shown.
6.4 Conclusion

It has been shown in this chapter that it is possible to differentiate between formalin fixed and live cell FT-IR spectra of the closely related leukaemic cell lines K562 and Nalm1 using the microfluidic device and buffer removal algorithm developed in CHAPTER 3 and CHAPTER 5. The fact that the differences between the four different data collection methods seem to be fairly comparable, shows that the buffer removal algorithm performed reasonably well under experimental conditions as well as on theoretical data. This was confirmed by the PCA comparison of the single fixed cell FTIR imaging data with that of the aqueous single cell pre and post buffer removal. Although the aqueous cell spectra after buffer removal cluster very closely with the fixed cell spectra obtained using FTIR imaging in PC1 there is a significant difference in PC2 in both cases. Although the algorithm is clearly able to remove buffer contributions and generate spectra of sufficient quality to differentiate between different cell lines whether it would be possible to classify smaller differences such as the same cell line treated with different drugs is still in doubt.
CHAPTER 7  Drug-cell interaction studies

7.1  Application of the microfluidic device to stem cell-drug interaction studies

The use of stem cells for regenerative medicine and for the generation of differentiated tissues is an area of particular scientific and medical interest. There are however, some key issues which need to be solved before these potentially revolutionary medicinal applications can be realised. The first of these challenges is how to maintain the stem cell phenotype within the population. A second is the generation of the correct fully differentiated cell population from the initial stem cell population in a reproducible and robust manner. The third challenge is to ensure the purity of the stem cell population [180-181]. One important reason for the careful monitoring of the differentiation process in stem cell populations is that because these cells are pluripotent they have the potential to form tumours once transplanted referred to as teratomas. This is a tumour that is composed of cells and tissue from the three germ layers (ectoderm, mesoderm and endoderm) [182]. Although there are currently methods available which can monitor the differentiation process either via the use of labelling or specific biomarkers these methodologies have been shown to have drawbacks [183-187]. In a recent study Clemens et al. used the TERA.cl.SP12 embryonal carcinoma derived cell line to study the effect of natural and synthetic retinoids on the differentiation of this cell line [69]. The study focused on the effects of TERA.cl.SP12 cell treatment with three different retinoids; the naturally occurring all trans-retinoic acid (ATRA) and two synthetic analogues 3-(5,5,8,8-tetramethyl-5,6,7,8-tetrahydronaphthalen-2-ylethynyl)benzoic acid (EC19) and 4-(5,5,8,8-tetramethyl-5,6,7,8-tetrahydronaphthalen-2-ylethynyl) benzoic acid (EC23) (Figure 7:1) [69].
The results of the Clemens et al. study were that it was possible to follow the progressive differentiation of the TERA.cl.SP12 over a period of 7 days using SR-FT-IR and FT-IR imaging. The effect of the different retinoids on differentiation could also be seen even between the EC23 and ATRA retinoids which both cause TERA.cl.SP12 to differentiate to neural cells [69]. This result was also found in a similar study by Maltman et al. who used MALDI-TOF MS (matrix assisted laser desorption ionisation time of flight mass spectrometry) to examine the proteomic profile of these cell under this culture conditions [188].

This previous work performed by Clemens et al. was however performed on formalin fixed cells whereas ideally this would be a live cell experiment [69]. With this in mind a preliminary experiment comparing fixed EC23 with DMSO treated control cells was done using the microfluidic device on the B22 beamline at the Diamond Light Source during this project. Due to the time restrictions associated with these SR-FT-IR experiments only one time point could be properly assessed. The 7 day time point was therefore chosen because at this time point the EC23 treated TERA.cl.SP12 were considered to be fully differentiated [69].
7.1.1 Methodology

The TERA.cl.SP12 cells for the aqueous cell study were cultured and fixed according to the protocol used by Clemens et al. and the SR-FT-IR data was collected and processed as previously state in 6.1 [69].

7.1.2 Comparison of the results for the fixed cells on CaF$_2$ substrates and that of the aqueous cell spectra

The PCA results of the fixed TERA.cl.SP12 cell spectra obtained from both the cytospun cells on CaF$_2$ substrates and those of the cells in PBS show separation along the PC2 axis (Figure 7:2). The loading plots also show common spectral features ~1650 cm$^{-1}$, ~1550 cm$^{-1}$ and 1470 cm$^{-1}$. The separation is however not perfect with a great deal of overlap between the two classes. A PC-LDA analysis was therefore used to determine if a greater degree of separation was possible as in Clemens et al. [69].

![Figure 7:2 PCA results for the fixed TERA.cl.SP12 cell spectra on both CaF$_2$ substrates (a) and c)) and fixed cells in aqueous solution (b) and d)). The DMSO treated control cells are shown in black and the EC23 treated cells in blue.](image-url)
The PC-LDA results also show a degree of separation in both aqueous and non-aqueous experiments. The loadings for both data sets also confirm that the separation is on similar chemistry with differences at ~1650 cm\(^{-1}\), ~1550 cm\(^{-1}\) and ~1470 cm\(^{-1}\) featuring strongly.

Figure 7:3 PC-LDA results for both fixed cell on CaF\(_2\) and the fixed aqueous cell spectra. The scores and loading plots for the non-aqueous cells are shown in a) and c) respectively whereas b) and d) depict the score and loading for the aqueous cells. DMSO treated cells are shown in black with the EC23 in blue.
In order to more closely examine the similarities and differences between the two methodologies a comparison of the mean 2\textsuperscript{nd} derivative spectra for each drug treated cell and the DF loading in each methodology was done. By comparing the results of these figures for each method a true evaluation can be done. Figure 7:4 shows how the two methods are broadly in agreement regarding the spectral features that are changing although there are some differences in the DF loading plots. This however is to be expected given the different experimental methods used and the fact that the PCs used to calculate the LDA will be slightly different.

**Figure 7:4** A comparison of the mean 2\textsuperscript{nd} derivate spectra for both DMSO (black) and EC23 (blue) treated cells and the DF1 loading (green) plot in each method. The results for the imaging experiment are shown in a) whereas those for the aqueous fixed cells are in b).
7.2 Proof of principle study for use of the microfluidic device in K562 doxorubicin (DOX) treatment FT-IR studies

Although groups such as Bellisola et al., Ceylan et al. and Le Gal et al. have previously investigated drug-cell interactions of a number of drugs with the K562 cell line, the aim of these studies was not, however, to determine the effect of low dose DOX treatment on the cell line [172, 189-190]. The focus of these studies was instead on determining how effective the drug treatment was, at either increasing the number of apoptotic cells observed as in Belliosla et al. or on determining the anti-proliferative effect of the drug as in Ceylan et al. [172, 189]. The work of Le Gal et al. did use a relatively low dose of DOX, however, the study was focused on the longer term generation of a drug resistant sub-cell line [190]. The investigation therefore was not interested in the short term effects as are studied here. However, in work performed by Morceau et al. and later by Szulawska et al. it has been shown that treatment of K562 cells with very low dose (40 nM) DOX can induce differentiation of these myeloid leukaemic cells along the erythrocyte differentiation pathway into more mature haemoglobin containing cells [191-192]. These groups found that treatment with DOX was able to induce slight differences over a 3 day period. In these studies, however, only a small percentage of 24 hr time point cells were found to exhibit differences from the untreated cells. The purpose of the investigation in this project was therefore to determine if differences could be detected at an earlier time point of 4 hrs and also to see if FT-IR could reproduce the differentiation results obtained during these earlier studies. The earlier studies also only sampled at 24, 48 and 72 hrs whereas in this experiment samples were also collected at 36 and 60 hrs in an effort to gain a more detailed picture of the differentiation process. As this differentiation experiment has not been investigated using FT-IR previously a standard fixed cell methodology was employed as a proof of concept. It was, however, necessary to determine whether any spectral differentiation detected could be due to an increase in apoptosis due to even this low level of DOX treatment. To prove this was not the case, trypan blue dye exclusion was performed and the results compared with those of the Szulawska et al. study [192].
7.2.1 Trypan blue dye exclusion study

The results of the trypan blue dye exclusion study show little difference in cell viability in the drug treated cells and the control cells from the same time point (Figure 7:5 a)). These findings are in keeping with the previously reported results of the Szulawska et al. study (Figure 7:5 b)) [192].

Figure 7:5 Cell viability study results for control (red) and DOX treated (blue) cells. a) shows the results from this study and b) the results from Szulawska et al. [192].
7.2.2 Results of the 72 hr time point

The PCA results of the 72 time point show some degree of separation between the DOX treated and control (untreated) cells (Figure 7:6 a)). The separation is mainly based on PC4 with the spectral differences at 1267 cm$^{-1}$, 1711 cm$^{-1}$ and 1725 cm$^{-1}$ being mainly responsible for the separation (Figure 7:6 c)). These spectral peaks correspond to changes in the nucleic acids (1267 cm$^{-1}$ and 1711 cm$^{-1}$) and lipids (1725 cm$^{-1}$) [142, 146].

Figure 7:6 PCA result for the 72 hr time point. The scores plot is shown a) with the loading plots for PC1 and PC4 in b) and c) respectively. The control cell spectra are shown in blue and the DOX treated cell spectra in red.
Although analysis of the underivatised data did produce a degree of separation, a second derivative transformation was performed. This was done for two reasons, the first of which was to try and extract further information about the separation of the two classes. The second reason was as a pilot study for a further investigation using the microfluidic device, it was important to establish that a similar data processing methodology as previously used in CHAPTER 6 would yield reasonable results. The PCA results for the second derivative data are shown in Figure 7:7. The loading plot for PC1 indicates that the separation of this data is based on spectral differences at 1691 cm\(^{-1}\), 1670 cm\(^{-1}\) and 1645 cm\(^{-1}\). These FT-IR bands correspond to changes in the protein Amide I indicating changes in the \(\beta\)-sheet (1670 cm\(^{-1}\) and 1691 cm\(^{-1}\)) ratio as well as differences in the amount of protein in an extended coil conformation (1645 cm\(^{-1}\)) [143, 152, 193].

![Figure 7:7 PCA results for the 2\(^{nd}\) derivative 72 hr time point. The scores plot is shown in a) and the loading plot for PC1 in b). The control cell spectra are shown in blue and the DOX treated cell spectra in red.](image-url)
In order to further study and understand this separation a plot comparing the mean second derivative spectra for both the DOX treated and the control sample sets as well as the loading plot for PC1 was constructed (Figure 7:8). This allows the smaller peaks contributing to the separation to be matched to differences in the spectra and so determine which of these are due to noise and which are due to changes in cell chemistry. It is now possible to see that the difference at $\sim 1655 \text{ cm}^{-1}$ is also a real spectral difference between the two classes. This corresponds to a change in the secondary structure of the protein content of the cell specifically in the $\alpha$-helix secondary structure [143]. In the Morceau et al. study they found that after 3 days there were significant differences in the protein chemistry of treated and untreated cells which is in agreement with the results presented here [191].

![Figure 7:8 comparison of the PC1 loading plot (green) and the mean 2nd derivative cell spectra for both DOX treated (red) and untreated control samples (blue).](image)
7.2.3 Results of the 60 hr time point

The PCA results for this time point are presented in Figure 7:9. The scores plot for this time point shows a more clear separation between the two classes than at 72 hrs, with most of the separation described by PC1. The loading plot for PC1 indicates that differences at 1716 cm\(^{-1}\), 1655 cm\(^{-1}\), 1483 cm\(^{-1}\), 1430 cm\(^{-1}\) and 1354 cm\(^{-1}\) are the reason for this separation. These bands correlate to changes in cellular chemistry of nucleic acids (1716 cm\(^{-1}\) and 1354 cm\(^{-1}\)), proteins (1655 cm\(^{-1}\)) and carbohydrates (1430 cm\(^{-1}\)) [143, 146, 152].

Figure 7:9 PCA results for the 60 hr time point. The scores plot is shown in a) and the loading plot for PC1 in b). The control cell spectra are shown in blue and the DOX treated cell spectra in red.
The PCA scores plot of the 2\textsuperscript{nd} derivative data show very good separation between the treated and untreated K562 cells along the PC1 axis (Figure 7:10). The loading plot for PC1 in this case is different to that of the underivatised data. The main difference between the two is that after 2\textsuperscript{nd} derivative trans cm\textsuperscript{-1} and 1645 cm\textsuperscript{-1} [143]. However there is still a strong nucleic acid contribution to the separation indicated by the peaks at ∼1554 cm\textsuperscript{-1} and ∼1410 cm\textsuperscript{-1} [146].

Figure 7:10 PCA result for the 2\textsuperscript{nd} derivative spectra 60 hr time point. The scores plot is shown in a) and the loading plot is shown in b). The control cell spectra are shown in blue and the DOX treated cells in red.
A comparison between the PC1 loading and the mean 2nd derivative spectra for the control and DOX treated cells after 60 hrs is shown in Figure 7:11. From this comparison the loading plot changes at 136 cm⁻¹, 1381 cm⁻¹, 1390 cm⁻¹, 1402 cm⁻¹, 1450 cm⁻¹, 1470 cm⁻¹, 1525 cm⁻¹ and 1568 cm⁻¹ can be assigned to visible differences between the mean spectra for each class. These band positions correlate to changes in the nucleic acids (1365 cm⁻¹, 1381 cm⁻¹, 1525 cm⁻¹ and 1568 cm⁻¹), proteins (1390 cm⁻¹, 1402 cm⁻¹ and 1450 cm⁻¹) and lipids (1470 cm⁻¹) [144, 146, 194-195]. It is also possible to assign the nucleic acid peak at ~1716 cm⁻¹ which although its contribution to the separation has been much reduced is still present.

Figure 7:11 comparison of the PC1 loading plot (green) with the mean 2nd derivative single cell spectra for the control (blue) and DOX treated (red) K562 cells.
7.2.4 Results of the 48 hr time point

The PCA results for the 48 hr time point also show very good separation between the two classes (Figure 7:12). The loading plot for PC1 in this case indicates that changes in nucleic acids (1714 cm\(^{-1}\) and 1490 cm\(^{-1}\)) and protein (1655 cm\(^{-1}\)) chemistry are the reason for this separation [143, 146].

Figure 7:12 PCA results for the 48 hr time point. The scores plot is shown in a) and the loading plot for PC1 in b). The control cell spectra are show in blue and the DOX cell spectra in red.
The PCA results of for the 2nd derivative however give a much more detailed picture of this separation (Figure 7:13). The loading plot of PC1 indicates that the separation of the two classes is based mainly on several protein peaks (~1670 cm\(^{-1}\), ~1655 cm\(^{-1}\) and ~1633 cm\(^{-1}\)) and the nucleic acid peak at ~1556 cm\(^{-1}\) [143, 146].

Figure 7:13 PCA results for the 2nd derivative spectra obtained after 48 hrs. The scores plot is shown in a) and the loading plot for PC1 in b).
A comparison of the PC1 loading plot and the mean 2\textsuperscript{nd} derivative cell spectra for both classes yielded a more detailed picture of the separation (Figure 7:14). This comparison confirmed the differences in the previously mention protein and nucleic acid bands however the smaller differences in lipid chemistry (~1504 cm\textsuperscript{-1}, ~1470 cm\textsuperscript{-1} and ~1465 cm\textsuperscript{-1}) as well as further differences in nucleic acid chemistry (~1527 cm\textsuperscript{-1}) were now highlighted [146-147, 152, 195].

![Figure 7:14 Comparison of PC1 loading plot (green) and mean 2\textsuperscript{nd} derivative single cell spectra for DOX treated (red) and control K562 cells (blue) after 48 hrs.](image)

The changes in protein and nucleic acids are consistent with those reported in both the previous studies by Morceau \textit{et al.} and Szulawska \textit{et al.} after 48 hrs of incubation with DOX [191-192]. The use of FT-IR however has also highlighted a difference in lipid chemistry which was not detected in either of the previous studies as the techniques and methodologies used were focused on specific protein and nucleic acid indicators of K562 differentiation [191-192].
7.2.5 Results of the 36 hr time point

The PCA results for the 36 hr time point show a good separation between the two classes (Figure 7:15). The separation described by the PC1 loading plot is due to changes in nucleic acids (~1714 cm\(^{-1}\) and 1358 cm\(^{-1}\)), proteins (~1655 cm\(^{-1}\)), lipids (~1477 cm\(^{-1}\)) and carbohydrates (~1430 cm\(^{-1}\)) [143, 146, 150, 152].

![PC1 loading plot with two classes of data](image)

Figure 7:15 PCA results for the 36 hr time point. The scores plot is shown in a) and the loading plot for PC1 is shown in b). The control cell spectra are shown in blue and the DOX treated cell spectra in blue.

The PCA results for the 2\(^{nd}\) derivative spectra also show good separation between the classes (Figure 7:16). The PC1 loadings show strong contributions for several protein bands (~1670 cm\(^{-1}\), ~1655 cm\(^{-1}\) and ~1645 cm\(^{-1}\)) and nucleic acids (~1555 cm\(^{-1}\)) [143, 146].
Figure 7:16 PCA results for the 2nd derivative cell spectra. The scores plot is shown in a) and the loading plot for PC1 in b). The control cell spectra are shown in blue and the DOX treated cell spectra in red.

The comparison of the PC1 loading plot with the mean 2nd derivative cell spectra for both classes is shown in Figure 7:17. This comparison highlights the smaller changes in nucleic acids (~1570 cm\(^{-1}\), ~1524 cm\(^{-1}\), 1483 cm\(^{-1}\) and 1381 cm\(^{-1}\)), carbohydrates (~1430 cm\(^{-1}\)) and lipids (~1470 cm\(^{-1}\) and 1444 cm\(^{-1}\)) that also contribute to the separation of these two classes [146, 152, 195].
7.2.6 Results of the 24 hr time point

The PCA results for this time point also show reasonable separation (Figure 7:18). The separation shown in this scores plot appears to be mainly based on PC3, however some contribution to the separation is from PC2. The loading plot for PC2 shows that this separation is based on the differences in nucleic acids (~1716 cm\(^{-1}\) and ~1465 cm\(^{-1}\)). The PC3 loading plot also shows changes in nucleic acids (~1707 cm\(^{-1}\), ~1720 cm\(^{-1}\) ~1490 cm\(^{-1}\), 1410 cm\(^{-1}\), 1356 cm\(^{-1}\) and 1261 cm\(^{-1}\)), protein (~1348 cm\(^{-1}\)) and lipids (~1728 cm\(^{-1}\)) [142, 146, 150].

Figure 7:17 comparison of mean 2\(^{nd}\) derivative single cell spectra for the control (blue) and DOX treated (red) K562 cells as well as the PC1 loading plot (green).
Figure 7:18 PCA results for the 24 hr time point. The scores plot is shown in a) and the loading plots for PC2 and PC3 are shown in b) and c) respectively.

As the separation of the PCA is more complex than at previous time points, PC-LDA was performed on the data set. The results of this are shown in Figure 7:19. The scores plot depicts reasonably good separation of the data. The loading plot of DF1 indicates that changes in nucleic acids (~1718 cm$^{-1}$, ~1490 cm$^{-1}$, ~1356 cm$^{-1}$ and 1259 cm$^{-1}$) and proteins (~1655 cm$^{-1}$ and ~1348 cm$^{-1}$) are the main source of separation for these classes [143, 146].
Figure 7:19 LDA results for the 24 hr time point. The scores plot is shown in a) and the loading plot for DF1 is shown in b).

To gain a more detailed understanding of the separation, 2nd derivative cell spectra were analysed by PCA (Figure 7:20). The loading plot for PC1 indicates that changes in protein bands (~1695 cm\(^{-1}\), ~1670 cm\(^{-1}\), ~1655 cm\(^{-1}\) and 1404 cm\(^{-1}\)), nucleic acids (~1630 cm\(^{-1}\), ~1535 cm\(^{-1}\) and 1365 cm\(^{-1}\)) and lipids (~1510 cm\(^{-1}\) and ~1470 cm\(^{-1}\)) are evident [143, 146, 152, 195].
The comparison of the mean spectra and the loading plot highlights some clear changes in chemistry that while present in the loading plot, were previously not obvious. A good example of this is the variation in the protein band at ~1545 cm\(^{-1}\) which is obviously a peak in both the mean spectra however in the loading plot this peak appears as a small trough and can easily be mistaken for a difference in nucleic acids at ~1556 cm\(^{-1}\) [146, 196]. There are also clear spectral differences at ~1450 cm\(^{-1}\) and ~1442 cm\(^{-1}\) which correspond to changes in lipid chemistry. These differences are also present in the loading plot however; they are easily missed as they appear on a sloping baseline [152, 156].
Figure 7:21 comparison of mean 2nd derivative spectra for DOX treated (red) and control (blue) plotted with the PC1 loading (green). The spectra were collected after 24 hr incubation with DOX.

The changes in protein and nucleic acid chemistry observed after 24 hrs incubation with DOX are consistent with the results of the previous studies. The differences in lipid chemistry however, were not reported in these works [191-192].

7.2.7 Results of the 4 hr time point

The PCA results for the 4 hr time point are particularly interesting as neither of the previous studies looked at such an early time point [191-192]. The scores plot show clear separation of the DOX treated K562 cells from the untreated control K562 cells (Figure 7:22). The loading plot for both PC2 and PC3 indicate the major changes in biochemistry at this early stage are mainly in the lipids (~1732 cm⁻¹) and nucleic acids (~1705 cm⁻¹) [142, 146].
Figure 7.22 PCA results for the 4 hr time point. The scores plot is shown in a) and the loading plots for both PC2 and PC3 are shown in b) and c) respectively. The control cell spectra are shown in blue and the DOX treated cell spectra in red.

As the separation of these two classes is based on two PCs, the use of PC-LDA should reduce the dimensionality of the separation into a single DF thereby simplifying the analysis. The PC-LDA scores plot shows reasonable separation of the two classes and the loading plot of DF1 indicates that the separation is still based on the changes in lipid and nucleic acid chemistry (~1730 cm\(^{-1}\) and ~1701 cm\(^{-1}\) respectively) [142, 146].
Figure 7:23 LDA of the 4 hr time point. The scores plot is shown in a) and the loading plot for DF1 in b). The control cell spectra are shown in blue and the DOX treated cell spectra in red.

However, there is also a significant sloping baseline in the DF1 plot which may indicate a physical effect such as scattering or changes in thickness are contributing to the separation. As the RMieS algorithm has been used to scatter correct this data, any effects of these changes on the physical effects of the spectra should be limited to a simple baseline offset which can be easily removed by performing a 2nd derivative transformation on the data. The results of PCA of this 2nd derivative data are shown in Figure 7:24. The scores plot for this data shows a great improvement in the separation of the two classes both in the overlap of the two groups and the fact that the separation can now be seen in PC1 indicating that a much higher percentage of the variance in the data set is actually describing discriminating chemistry. The loading plot of PC1 indicates that the separation is due to changes in nucleic acid (~1709 cm⁻¹, ~1638 cm⁻¹, ~1622 cm⁻¹ and ~1610 cm⁻¹) and protein (~1695 cm⁻¹, ~1670 cm⁻¹ and ~1550 cm⁻¹) chemistry [143, 146, 150, 197].
Figure 7: 24 PCA of the second derivative data. The scores plot is shown in a) and the loading plot for PC1 in b). The control cell spectra are shown in blue and the DOX treated cell spectra in red.
In order to help assign the more closely packed bands in the PC1 loading plot a comparison of the loading plot and the mean 2nd derivative spectra for the DOX treated and untreated K562 samples was done (Figure 7:25). This confirmed the spectral assignments of the peaks at ~1709 cm\(^{-1}\), ~169 cm\(^{-1}\), 1670 cm\(^{-1}\), ~1638 cm\(^{-1}\) and ~1610 cm\(^{-1}\) as previously stated. However the peaks seen at ~1622 cm\(^{-1}\) and 1552 cm\(^{-1}\) on the PC1 loading plot in Figure 7:24 previously assigned to changes in nucleic acids do not appear to correspond to differences between the mean spectra at the same wavenumber. There are however small spectral differences at the apex and base of peaks at ~1630 cm\(^{-1}\) and ~1547 cm\(^{-1}\) and it is these spectral differences which correspond the PC1 loading peaks. This would not affect the assignment of the peaks at ~1622 cm\(^{-1}\) and ~1630 cm\(^{-1}\) as this is still attributed to nucleic acid chemistry. However the peak at ~1552 cm\(^{-1}\) is a nucleic acid vibration whereas the peaks at 1547 cm\(^{-1}\) where the spectral differences occur, is a protein vibration [145-146].

![Figure 7:25 comparison of mean 2nd derivative spectra for DOX treated (red) and control (blue) plotted with the PC1 loading (green).](image)
7.2.8 Comparison of the different time points

The results of the FT-IR analysis have so far shown that it is possible to separate the DOX treated K562 cell spectra from the K562 control cell spectra at each individual time point. However the aim of this study was to determine whether or not it was possible to follow the DOX induced differentiation of K562 cells. With this in mind a comparison of the 2<sup>nd</sup> derivative spectra for both the DOX treated and control samples at each time point was done (Figure 7:26). The result of this comparison was however slightly disappointing in that the only consistent variation appears to be in the amide III protein band at ~1280 cm<sup>-1</sup> (Highlighted in yellow on Figure 7:26). The amide III band is very elevated in the 4 hr and 24 hr DOX treated spectra. The 36 hr time point however shows only a small increase in the amide III and after 48 hrs of DOX treatment the amide III increase is even smaller. By 60 hrs of DOX treatment the amide III has been reduced to the same levels as in the untreated control cells.
Figure 7: A comparison of the mean 2nd derivative spectra for each time point. The DOX treated cell spectra are shown in red and the control spectra in blue. The spectra for 72 hrs are shown in a), 60 hrs in b), 48 hrs in c), 36 hrs in d), 24 hrs in e) and 4 hrs in f).
As the comparison of the loading plots for the individual PCA of each time point separately did not appear to offer any obvious trend in the data, a final PCA was performed. This PCA used all the control K562 spectra from each time point as a single group and then compared each DOX treated K652 time point to it. The results of the PCA are shown in Figure 7:27. The scores plot shows that there is not really a gradual movement of the DOX treated groups across the PCA space as would be expected if the separation was due to a gradual change in differentiation state of the K562 cells upon DOX treatment. It does appear however that after 48 hrs of DOX treatment something does change as there is an increase in the α-helical protein chemistry and a decrease in β-sheet protein chemistry [143, 198].

Figure 7:27 a) PCA scores plot the K562 control cell spectra are shown in blue, spectra after 4, 24, 36, 48, 60 and 72 hrs DOX treatment are shown in red, green, orange, magenta, turquoise and black respectively. The loading plot for PC2 is also shown in b).
7.2.9 Conclusion

The results of the FTIR analysis previously discussed in this chapter clearly show that single cell FTIR spectra pre-processed as in CHAPTER 6 are able to distinguish K562 cells treated with DOX from those which have not been DOX treated from drug incubation times as early as 4 hrs. This illustrates that further experiments using live cells treated with DOX could be performed using the microfluidic device and FTIR analysis. However, due to the time limitations of this project, this could not be performed here.

The results of the FT-IR analysis previously discussed in this chapter clearly show that single cell FT-IR spectra pre-processed as in CHAPTER 6 are able to distinguish K562 cells treated with DOX from those which have not been DOX treated from drug incubation times as early as 4 hrs. As both of the previous studies only detected differences in the cell populations after 24 hrs and also only in a small proportion of the cell population, this shows FT-IR is a powerful tool for observing drug-cell interactions [191-192]. The result of the comparison between the different time points is however disappointing as the comparison does not appear to show any real consistent spectral differences over the time course as would be expected with this kind of study. The differences in the amide II band do however suggest there may be some differential shift upon DOX treatment, however a larger scale study using FT-IR imaging should be performed as this would allow the generation of a larger data set which would allow a more robust analysis to be performed.
CHAPTER 8  Discussion and future work

8.1  Design and manufacture of the microfluidic device

During this project a microfluidic device capable of obtaining both aqueous live cell and fixed cell spectra using FTIR has been developed. The design and microfabrication of this microfluidic device has been described in CHAPTER 3. Although the design outlined and tested in this thesis is a reasonable prototype, in order to improve the device and move further towards a high through-put system a number of improvements to the device need to be made. The first of which is to test a few different sealing materials as recent testing has revealed that the seal on the intake side of the channel weakens after prolonged usage as marked in Figure 8:1. This may be due to the highly hydrophobic nature of the CaF$_2$ substrate to which the sealing film is adhered to. Although this seal is supported with epoxy resin, both of these materials are usually used for plastic devices and it may be that a more specialised hydrophobic epoxy resin or even the use of silicon rubber may increase the lifespan of this seal.

Figure 8:1 schematic of device with leakage area highlighted.

Point of exit
Weak seal
This has not been tried as until recently the leaks appeared to stem from problems with the bonding of the analysis chamber. However on the most recent beamtime visit, it was possible to observe a progressive leak which interestingly followed the CaF$_2$ seal boundary before exiting at the side (also marked on Figure 8:1). The use of a more hydrophobic sealant may help relieve this problem.

Another issue with this device is that although it is possible to flow cells through the device when the flow is paused. In order to collect spectra, many of the cells then become stuck on to the lower of the CaF$_2$ substrates when the flow is resumed. One possible way to alleviate this would be to work in a continuous flow mode. This would however require the addition of a cell detection module to the device. A commonly used technique for this is light scattering. In order to trigger data collection at the appropriate point the direct communication of the output from this device with the FPA and bench FT-IR would be necessary. One possible method of inserting a light scattering module would involve the placement of a fibre optic cable in to the device perpendicular to the main channel and located about halfway up the ramp leading in to the analysis chamber as shown in Figure 8:2.

![Figure 8:2 schematics of the possible position of the optic fibre for the light scattering module](image)
A green laser source is attached to one end of the cable and a Photomultiplier tube (PMT) at the other. This detector is ideally suited to the rapid detection of changes in light intensity particularly at low levels of incident light such as are likely in this application [199]. A green laser would be used as the PMTs currently available within the work group are more sensitive to photons in the green spectral region than the red spectral region [200]. The light scattering module could be used as a switch to activate the FT-IR instrument. When a continuous beam of light is emitted from the laser source and reaches the PMT detector a constant signal would be detected. As a cell passes across this beam incident light would become scattered and a drop in intensity detected would result such as described in Figure 8:3.

![Figure 8:3](image)

**Figure 8:3** a) A cell approaching the laser beam in the light scattering module, b) the cell passing through the laser beam and c) the cell leaving the light scattering module and approaching the FT-IR analysis chamber. d) The predicted absorbance output from the light scattering module as the cell travels through the system.

This drop in intensity could be used for two purposes. The first would be to initiate the FT-IR spectrometer and FPA. This however leads on to another problem which would need to be solved before this type of system could be used effectively. The problem is that the software associated with the Varian FT-IR imaging system requires a minimum of 4 co-scans to be collected at a time. As the cell is in flow...
across the detector the pixels which contain cell chemistry will therefore move between co-scans leading to a dilution of the already weak cell signal. In order for this to become a viable option a way to access each co-scan is required. The pixels containing the cell spectra could then be averaged together to yield a single high quality spectrum for buffer removal and further analysis.

The second purpose is that the drop in intensity at the detector would be directly proportional to the size of the scattering particle, in this case the cell [201]. This would also allow the exact size of the cell for which the FT-IR spectrum is being collected from to be obtained. It might therefore be possible to collect spectra from several different cell types in a single sample and classify the cell spectra in to the relevant cell types, as well as detecting any cells within each cell class that are not spectrally normal i.e. are cancerous.

It would also be necessary to alter the current microfluidic channel system to a more complex microfluidic system in the outer section of the device as the current arrangement does not guarantee that only one cell is present in the analysis chamber at any one time. The more simplified system was used for the preliminary testing as performed during this project because the flow was stopped during the analysis and therefore it was possible to move the FT-IR focal point to the position of the cell. In a high through-put flow system this is not possible. A schematic of the proposed changes is presented in Figure 8:4.

Figure 8:4 Schematic of the proposed changes to the microfluidic section of the device.
The introduction of a cell separating stream allows the cells to be clearly separated from one another by fluid plugs. In this application an oil solution would be the most likely choice as oils are immiscible in water based buffer solutions such as those used here.

### 8.2 Future applications of the microfluidic device

Chapters 6 and 7 describe a number of applications to both fixed and live cell studies performed using the microfluidic device. These studies have illustrated that it is possible to obtain spectra of a suitable quality using this microfluidic device. Therefore we can conclude that the design and microfabrication of this device has been successful. The device therefore could also be utilised for further cell studies.

The first of these would be to perform a live drug-cell interaction study as a comparison to the K562 DOX study conducted in section 7.2. Although results from this study did not give rise to the hoped for consistent differences in cell chemistry over the time periods studied which may have been associated with differentiation, we were able to detect changes in amide III chemistry following DOX treatment. Therefore, further studies investigating the cellular response to DOX treatment over the first 24 hrs more closely could be performed as this has not previously been studied and may yield more differentiation related changes in chemistry than the current study performed in section 7.2. Another set of studies that would be interesting to conduct are a live and companion fixed drug-cell interaction study using the Nalm1 cell line and DOX. This would be interesting because DOX treatment of this cell line has previously been shown to induce partial cell differentiation by Martin-Kleiner et al. using similar methods to those of Morceau et al. and Szulawska et al. [191-192, 202].

The work with the TERA.cl.SP12 stem cell line would also be worthy of further examination. The first set of experiments which could yield interesting results would be to look at fixed cells collected at earlier time points in an aqueous environment to see if it would be possible to detect differences in cell chemistry similar to those found in the cytospun fixed cell samples in the Clemens et al. study [69]. It would also be interesting to conduct a live cell study using a similar methodology to that discussed in section 7.1 and extend this over the earlier time points. This would then allow a comparison between the live and fixed cell results across the whole cell
differentiation process and help determine if the fixation process has any detectable effect on the results.

Another experiment which could lead to a useful application of the fully functioning flow system would be to try fixed cells of a number of prostate and bladder cancer cell lines such as those used by Harvey et al. to determine if it would be possible to classify them [3]. By using the light scattering data to determine cell size and the aqueous cell spectra to determine the cell chemistry this should be possible.

In conclusion, the microfluidic device developed during this project has demonstrated its suitability to a wide range of cell based applications. Spectra of the desired quality have been collected using FTIR in conjunction with this microfluidic device. Therefore, the desired aim of this project, to design and develop a microfluidic device capable of obtaining live cell spectra of a suitable quality for FTIR analysis has been achieved.
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