Infrared Spectroscopy to Monitor Drug Response of Individual Live Cells

A dissertation presented by

Ellen J. Marcisin

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ABSTRACT OF DISSERTATION

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ABSTRACT

A patient diagnosed with cancer needs a treatment option that proposes the best prognosis. Most treatment options include chemotherapy, but the prediction for how the chemotherapeutic agents will react with the patient’s cancerous cells is based on previous outcomes of patients with similar cancer and conditions. This approach is somewhat flawed, since not every person with the same type of cancer responds to a drug with the same effectiveness in the same way; similarly, there is not one universal drug for the treatment of different cancer types. Often the testing of several treatment plans, or combinations of drugs, is necessary to develop a regimen that an individual responds to and this simultaneously compromises a patient’s already unstable health. Chemotherapeutic treatments can be monetarily, psychologically, and physically demanding, introducing a need for a methodology that would accommodate the pre-screening of potential drug treatment options on an individual’s diseased cells prior to administration of potentially ineffective and harmful drugs to the body. Presented are efforts toward introducing infrared (IR) spectroscopic means as a tool to develop personalized cancer treatment plans by monitoring the response of live cells to chemotherapeutic agents in vitro.

The methods and results presented utilized Fourier transform infrared (FTIR) microspectroscopy to monitor IR spectroscopic response of live cells. Data presented here demonstrated the feasibility of using an in-lab designed live sample chamber capable of sustaining cellular life for long experimental times, including a 24 hour experiment following the same cells, which represents the first FTIR live cell study to be published for that experimental duration. Also presented are data demonstrating the ability to detect drug induced spectral changes in HeLa cells exposed to a commonly used antineoplastic agent for various cancers,
cyclophosphamide monohydrate, in comparison with cells kept under normal culture conditions. Principal component analysis, a multivariate analytical technique, was employed to demonstrate subtle, yet distinct biochemical changes observed for all data collected. Most recent results were obtained after optimization of the methodology, in conjunction with newly developed MATLAB based algorithms, allowing faster data acquisition, better spectral quality and noise reduction.
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I dedicate this work in memory of my grandfather, an important person who influenced so many people, including myself, with his knowledge, love, and compassion.

Theodore A. Olsson Jr.

Northeastern University, Class of 1953

*June 25, 1930 – August 18, 2008*
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<thead>
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<th>Abbreviation/Symbol</th>
<th>Meaning</th>
</tr>
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<tbody>
<tr>
<td>.fsm</td>
<td>Perkin Elmer, Inc. raw image file format</td>
</tr>
<tr>
<td>.sp</td>
<td>Perkin Elmer, Inc. raw single beam file format</td>
</tr>
<tr>
<td>$A$</td>
<td>Absorbance</td>
</tr>
<tr>
<td>$\bar{A}$</td>
<td>Mean Absorbance</td>
</tr>
<tr>
<td>ACS</td>
<td>American Cancer Society</td>
</tr>
<tr>
<td>ARO</td>
<td>All Reflecting Objective</td>
</tr>
<tr>
<td>bkgd</td>
<td>background single beam spectrum</td>
</tr>
<tr>
<td>BSS</td>
<td>Buffer Saline Solution</td>
</tr>
<tr>
<td>$C$</td>
<td>Concentration</td>
</tr>
<tr>
<td>C1</td>
<td>Condenser 1</td>
</tr>
<tr>
<td>CaF$_2$</td>
<td>Calcium Fluoride</td>
</tr>
<tr>
<td>CellAbs</td>
<td>Cell absorbance spectrum</td>
</tr>
<tr>
<td>cm$^{-1}$</td>
<td>Wavenumber</td>
</tr>
<tr>
<td>$d$</td>
<td>bond distance</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EM</td>
<td>Electromagnetic radiation</td>
</tr>
<tr>
<td>$E_n$</td>
<td>Energy</td>
</tr>
<tr>
<td>$F$</td>
<td>Force exerted</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FT</td>
<td>Fourier Transform</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier Transform Infrared</td>
</tr>
<tr>
<td>$G$</td>
<td>Diffraction Grating</td>
</tr>
<tr>
<td>GUI</td>
<td>Graphical User Interface</td>
</tr>
<tr>
<td>HeNe</td>
<td>Helium Neon</td>
</tr>
<tr>
<td>$H_n(y)$</td>
<td>Hermite nth polynomial</td>
</tr>
<tr>
<td>$I(\lambda)$</td>
<td>Light transmitted</td>
</tr>
<tr>
<td>$I(\lambda_0)$</td>
<td>Incident light</td>
</tr>
<tr>
<td>IlluminatIR</td>
<td>Smiths Detections IlluminatIR infrared microscope</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared</td>
</tr>
<tr>
<td>$J(x)$</td>
<td>Time domain interferogram</td>
</tr>
<tr>
<td>$k$</td>
<td>Spring constant</td>
</tr>
<tr>
<td>$l$</td>
<td>pathlength</td>
</tr>
<tr>
<td>L1</td>
<td>Lens 1</td>
</tr>
<tr>
<td>L2</td>
<td>Lens 2</td>
</tr>
<tr>
<td>low-e</td>
<td>Low emissivity</td>
</tr>
<tr>
<td>$m$</td>
<td>mass</td>
</tr>
</tbody>
</table>
M1  Mirror 1
M2  Mirror 2
MCT  Mercury Cadmium Telluride
MIR  Mid Infrared
N  Normalization Constant (Chapter 2), Fringe Number (Chapter 4)
NA  Numerical Aperture
NA-PC  Noise Adjusted Principal Component
PBS  Phosphate Buffer Solution
PC  Principal Component
PCA  Principal Component Analysis
PE 400  Perkin Elmer Spotlight 400 Series infrared microscope
rawcell  Single beam spectrum of a raw cell
S(v)  Frequency domain interferogram
S/N  Signal to Noise
S1  Slit 1
S2  Slit 2
SF1  Scaling Factor 1 (Water)
SF2  Scaling Factor 2 (Calcium Fluoride)
t  Time
T  Transmission
V  Potential Energy
x  Distance
α  Incident angle
β  Angle of diffraction
ε  Molar absorptivity
λ  Wavelength (nm)
μ  Reduced Mass
φ_e  Excited state wave function
φ_g  Ground state wavefunction
ω  Frequency
Chapter 1: Introduction to Vibrational Spectroscopy for the Analysis of Individual Live Cells

1.1 Introduction: Current Problem with The Chemotherapeutic Treatment of Cancer

Chemotherapeutic treatment of cancer can often be debilitating, both financially and physically, and is often as taxing as the disease itself. In 2009 the American Cancer Society (ACS) sponsored a report to investigate the issue of bankruptcy among cancer patients and survivors. The ACS helpline was overwhelmed with calls from desperate people who couldn’t afford costly treatments, which resulted in the collection of debt that they had no chance of surmounting. The report stated that during 2008 there were ca. 684,450 new cancer diagnoses for people under the age of 65 in the US and of that number only 70% carried insurance. Many of the callers who were covered by insurance still were forced into debt. One example was a ten year old girl undergoing treatment for leukemia. Under her parents’ insurance, she was about to exceed her lifetime allowed coverage at the age of 10 while still undergoing treatment and was not going to be able to continue to cover the costs. There were several cases like this one for all age groups.

It is often the case that drug response can not be thoroughly evaluated until the drug is administered to the patient. Prior to knowing the patient’s own response, treatment plans are developed based on the outcome of previous patients with similar cancer. The major flaw in that approach is that not every patient or cancer reacts to the same drugs and there is no universal drug for the treatment of all cancers. Therefore, methodologies that would allow for the efficacy screening of chemotherapeutic drugs in vitro before administration to a patient would be a great advancement in the field of oncology. This would be beneficial because it would save the patient
the aggravation of going through potentially painful, toxic treatments, which may be unnecessary, in the quest for a treatment to which the cancer responds. Since most of these drugs are cytotoxic, pre-screening would also help prevent further deterioration of the patient’s already compromised health. Maintaining health and strength can be essential to a patient’s survival.

Cancerous cells are typically identified via morphological abnormalities, even though biochemical changes have begun to occur long before the cells exhibit visible change. Fourier Transform Infrared (FTIR) microspectroscopy has demonstrated itself as a useful diagnostic tool with the ability to detect disease related spectral changes prior to morphological changes.\textsuperscript{2-5} It has not yet been recognized for its potential role as a tool to pre-screen chemotherapeutic agents outside of a patient’s body. In the development of treatment plans, it would be beneficial to have a methodology that could provide information specific to an individual’s cancer and have the ability to predict response.

1.2 How FTIR Microspectroscopy Contributes to Medical Diagnostics

Fourier Transform Infrared (FTIR) microspectroscopy was demonstrated to be a potentially useful diagnostic tool over the last two decades with the ability to detect disease related spectral changes prior to morphological changes in cells and tissue.\textsuperscript{2-7} FTIR microspectroscopy has been used successfully to differentiate individual cells based on cell cycle, cell division and quiescence; different cellular types such as cells from different anatomical regions, or cells of the same region but from different species; cell maturation; cellular death; and cells that are healthy compared to those which are considered diseased.\textsuperscript{5, 6, 8-16} FTIR microspectroscopy of cells and tissues has gained significant recognition in these areas, as
there are several international conferences that meet annually (and bi-annually) to discuss, debate, and share results in this growing area of interest.

Early live cell microspectroscopic research was reported by Miljković et al., and Moss et al., and more recently by Tobin et al.17-19 Both demonstrated that live cell experiments were feasible by performing short term spectroscopic measurements on unfixed, single cells in aqueous culture medium or buffer solution.17, 18 Live cell experimentation is confronted with an obstacle presented by strong water absorption bands (namely the water deformation band at ca. 1620 cm⁻¹) in the spectral region of cellular proteins which creates an abnormal-looking band profile. The abnormal band profile and the issue of liquid water will be thoroughly discussed in Chapter 3 of this dissertation. Shown in Figure 1.3 are spectra collected in (A) aqueous conditions, demonstrating the abnormal band profile and (B) a spectrum illustrating the typical band profile of fixed or dried cells.

More recently there has been a gradual shift in efforts to focus on performing IR spectroscopic measurements on live cellular systems.10, 16, 19, 20 Much can be learned from fixed clinical and laboratory cultured samples; however, being able to perform real time live studies initiates the possibility to expand on the use of this technique as a diagnostic tool and promote its use for potential roles in the evaluation of disease treatments. There is no better solution for understanding a living system than to have the ability to probe that system in vivo.

The work presented in this dissertation advances the possibilities for live cell experimentation by demonstrating acquisition of data over extended experimental times and a progression of acquisition technique which resulted in an optimized methodology for obtaining IR data for live cells in liquid culture medium. Presented in this dissertation are results
Figure 1.1 Abnormal Cellular Band Profile
Spectrum of an individual live cultured HeLa cell collected in aqueous medium (A) in comparison with the typical spectrum observed for fixed or dried cells (B, two examples shown in Blue and Red). The overcompensation for the water deformation mode at 1620 cm\(^{-1}\) creates an abnormal looking band profile in the protein region where the amide I frequency band around 1650 cm\(^{-1}\) appears equal in intensity to the amide II frequency band.
monitoring the induced spectral changes occurring on the biochemical level of live HeLa cells as a result of perturbations imposed on the cellular environment.

1.3 Scope of This Dissertation

Presented are efforts toward introducing an FTIR microspectroscopic methodology as a tool to develop personalized cancer treatment plans. FTIR provides the ability to monitor the response of live cells to chemotherapeutic agents in vitro. The research presented in this dissertation includes the design of a functional sample chamber able to facilitate the collection of FTIR microspectroscopic measurements to monitor induced spectral changes over extended experimental periods. The research presented represents the fundamental foundation for a methodology to pre-screen chemotherapeutic drug options against a patient’s cancerous cells without exposing the patient to potentially toxic, ineffective drugs in an effort to find a treatment that presents a positive prognosis.

Chapter 2 of this dissertation presents an introductory overview of vibrational spectroscopy and the reasons IR microspectroscopy was chosen for interrogating the response of individual live cells to potential drug treatments. Included is a detailed explanation of the instrumental components necessary for obtaining IR data and the origin for the observed vibrational modes exhibited by the biomolecules of subcellular components.

Chapter 3 presents a detailed description of the experimental conditions and designs specific to the research conducted for this dissertation. Culture conditions and sample preparation were uniform for all data collected. Experimental designs include the various stages of development for the live cell sample chamber created to facilitate microspectroscopic measurements of individual live cells in liquid culture medium. Also provided is a thorough comparison of the data collection methods utilized specific to the Perkin Elmer Spotlight 400
Series (PE 400) IR microscope. The PE 400 infrared microscope is capable of two different types of data collection modes; point mode, which was used in all initial experimentation and image mode, which was used in an optimized approach. Also included in Chapter 3 is a MATLAB based liquid water correction algorithm designed to address the abnormal band profile observed with collection of cells in liquid culture medium.\textsuperscript{21}

Following the experimental conditions and designs are results obtained using initial point mode data acquisition methodologies. The results, presented in Chapter 4, illustrate the ability to monitor induced spectral changes in individual live cells as a result of perturbations applied to the cellular environment. The results include experiments illustrating the ability to keep individual cells alive for the longest FTIR microspectroscopic study (+24 hours) performed in the literature.\textsuperscript{21}

The initial point mode data acquisition was extremely time prohibitive and labor intensive. Presented in Chapter 5 using image mode data acquisition methodologies are results illustrating an optimized technique for data acquisition. Optimization was achieved by utilizing image mode collection in conjunction with MATLAB based algorithms, allowing acquisition of larger datasets in less time, with a better signal to noise ratio and improved spectral quality.

Results presented in Chapters 4 and 5 demonstrate the potential this type of technique provides for pre-screening chemotherapeutic treatments by illustrating experiments designed to detect and evaluate individual live cellular response to a commonly used chemotherapeutic drug, cyclophosphamide monohydrate. Chemometric analysis, specifically principal component analysis (PCA), was utilized to detect and illustrate biochemical changes occurring as a result of the drug treatments.
The conclusions of the research presented in this dissertation and a summary of all methods and results are in Chapter 6. Also presented are future directions for this research. The potential for this technique as a tool in the pre-screening of potential drug treatments is in the developmental stages. In the long term, this type of work could help improve the outcome and quality of life of cancer patients and cancer survivors by preventing the unnecessary, expensive experimentation of drug treatments in the body. The population of people affected by cancer increases every year. It is time to improve the practices used to treat the disease.

1.4 References


14. Boydston-White, S.; Romeo, M.; Chernenko, T.; Regina, A.; Miljkovic, M.; Diem, M., Cell-cycle-dependent variations in FTIR micro-spectra of single proliferating HeLa cells:


Chapter 2: Fundamentals of Vibrational Spectroscopy for the Analysis of Individual Live Cells

2.1 Introduction to Vibrational Spectroscopy

Infrared spectroscopy is a technique of vibrational spectroscopy in which the absorption of infrared light by molecular matter is observed. It is ideal for studies of eukaryotic cells because the IR spectrum of cellular components represents a molecular fingerprint; thus, the spectrum of a cell represents a superposition of all cellular components. The wavelength of an IR photon is on the same order of magnitude as cells and sub-cellular material, between 2.5 and 25 μm. Provided here is the background and theory essential to the understanding of how FTIR instrumentation and technique can be used to interrogate live diseased cells in an effort toward improving medical practices for the evaluation and treatment for disease.

Spectroscopy is used to probe the discrete energy levels inherent to all atomic matter and molecular systems. Energy levels are populated by illumination with electromagnetic radiation (absorption) or by interactions (i.e. collision, scattering) with other atomic or molecular systems. The interaction of electromagnetic radiation (EM) with matter is governed by quantum mechanics, which utilizes mathematical models to develop explanations for the interactions of matter with light. Quantum mechanical models are developed to describe the energy level structure of atoms or molecules and allow information to be gained from the acquired spectra regarding structure, functional groups and dynamic properties based on the inherent vibrational motions of the atoms.1

EM radiation (i.e. light) is a broad term and be can further classified based on wavelengths. Figure 2.1 illustrates the EM spectrum with various objects for comparison of the
Figure 2.1 The electromagnetic spectrum
In this depiction of the electromagnetic spectrum, the wavelengths progress from longer (left) to shorter (right), with objects for size comparison below. For example, radio waves can be equivalent to the length of a football field and are monstrously longer than that of the atomic radius of the Lithium atom (152 pm). A eukaryotic human cell is roughly the wavelength size equivalent of a mid-IR photon.
different wavelengths. EM radiation is composed of oscillating electric and magnetic fields that travel through space as a harmonic wave. Harmonic waves are used to describe how IR light interacts with matter. The interaction mechanism between an IR photon and observed molecular motions depends on the change in the dipole moment of the molecule with the vibrational motion. An introduction to harmonic motion is included in the subsequent Sections of this Chapter. Also included in this Chapter is a detailed account of the components that are employed in FTIR instruments, one of which is the source. This is particularly important because all data presented in this dissertation were acquired using a commercially available source rather than a synchrotron beam source, which is claimed several times in the literature as the only way to achieve acceptable S/N for live cell experiments.\(^2-8\)

Vibrational transitions can be observed for polar molecules because polar molecules strongly absorb IR radiation. Water is the most polar molecule and so its absorption presents a large obstacle for IR spectroscopic measurements. Water presents strong absorption in the fingerprint region of cells, distorting the observed frequency band intensities. A detailed account of water contributions is included in subsequent Sections of this Chapter.

2.2 IR Spectroscopic Theory

2.2.1 Dipole Moment

All molecules that exhibit a dipole moment are susceptible to the force of an electric field caused by EM radiation and will exhibit IR transitions. An electric dipole moment is the physical observable calculated by the summation of all electrons and nuclei of a molecule by the charge of atoms and electrons \((q_i)\) and the positions \((r_i)\) as described by Equation 2.1.\(^9\)

\[
\mu = \sum_i q_i r_i
\]
Absorption occurs due to a net change of dipole moment for polar molecules and as a result the molecule experiences resonant vibration. The transition moment, as defined in Equation 2.2 in one dimensional space, determines whether or not a transition is allowed and the intensity of the transition. It could also be interpreted as the force the electric field exerts on the sample molecule; a transition can only occur if the transition dipole moment is non-zero. The expectation value of the transition dipole moment equals the integral over the product of excited state wave function $\phi_e$, the transition moment operator and the ground state wave function $\phi_g$.\(^9\)

\[
\text{Equation 2.2 } \mu = \int \phi_e \hat{\mu} \phi_g dx
\]

The wave functions, to be discussed in the subsequent Section, are the solutions of the vibrational Hamiltonian and are referred to as the harmonic oscillator wave functions.

### 2.2.2 Harmonic Oscillator

Motion of atoms can be visualized with the classical example of harmonic motion where a weight is suspended by a spring, Figure 2.2. A weight with mass $m$ on a spring oscillates about its equilibrium position. The restoring force $F$ in harmonic motion is given by Equation 2.3, where $k$ is the force constant of the spring in terms of force/ distance (N/m) and $x$ is the displacement from the equilibrium position. Equation 2.3 is known as Hooke’s Law.

\[
\text{Equation 2.3 Hooke’s Law } F = -kx
\]

The equation of motion can be set up as the follows:

\[
\text{Equation 2.4 } \frac{d^2x}{dt^2} = -\frac{kx}{m}
\]
Figure 2.2 Harmonic Oscillator
The classical example for harmonic motion is a mass or a weight suspended from a mounted spring.
This equation can be solved by using the trial function shown in Equation 2.5 to obtain the displacement, \( x \), as a function of time, \( t \), where \( A \) is the amplitude, \( \omega \) is frequency and \( b \) is a constant.\(^9\)

Equation 2.5 \( x(t) = A \sin(\omega t + b) \)

Using the expression from Equation 2.5, Equation 2.4 becomes the following Equation 2.6, which demonstrates that the frequency is independent of the amplitude and depends only on the mass and the strength of the spring.

Equation 2.6 \( \omega^2 A \sin(\omega t + b) = \left( \frac{k}{m} \right) A \sin(\omega t + b) \)

\[\omega = \sqrt{\frac{k}{m}}\]

The potential energy of the system for the harmonic oscillator is obtained by integrating Equation 2.3, and is described as:

Equation 2.7 \( V = \frac{1}{2} kx^2 \)

Equation 2.7 describes the shape of the parabolic curve demonstrated in Figure 2.3, the potential energy curve. Whereas the classical system does not exhibit quantized energy levels, the quantum mechanical harmonic oscillator does. Equation 2.8 gives the vibrational Schrödinger equation and the solutions for Equation 2.8 result in the allowed energy levels are described by Equation 2.9. In this Equation, the form of the potential energy is unchanged from the classical case, whereas the kinetic energy contains the quantization of the linear momentum. In Equation 2.8, \( \hbar = \frac{\hbar}{2\pi} \) and \( n \) represents the quantum numbers \( n = 0, 1, 2, 3...^9 \) \( E \) is the energy and \( \mu \) represents the reduced mass of two atoms bonded together, calculated by Equation 2.10.\(^9\)
Figure 2.3 Harmonic Oscillator Potential and Energy Diagram
The boundaries of the quantum mechanical harmonic oscillator are defined by the potential energy. Within the harmonic approximation, the potential energy is represented by a parabolic curve.
Equation 2.8 Vibrational Schrödinger Equation
\[-\frac{\hbar^2}{2\mu} \frac{d^2}{dx^2} \phi + \frac{1}{2} kx^2 \phi = E\phi\]

Equation 2.9 Vibrational Energy Levels
\[E_n = \left( n + \frac{1}{2} \right) \hbar \sqrt{\frac{k}{\mu}}\]

Equation 2.10 Reduced Mass
\[\mu = \frac{m_1 m_2}{m_1 + m_2}\]

The lowest energy state, the ground state, at \( n = 0 \), is referred to as the zero point energy and is equivalent to \( \frac{1}{2} \hbar \nu \) where \( \nu \) represents \( \frac{1}{2\pi} \sqrt{\frac{k}{\mu}} \). The wave functions resulting from Equation 2.8 take on the form of Equation 2.11 where \( N \) is the normalization constant and \( H_n(y) \) is the \( n^{th} \) Hermite polynomial in the variable \( y \). The Hermite polynomials for \( n = 0 \) to 4 are shown in Table 2.1.

Equation 2.11
\[\phi(x) = NH_n(y)e^{-\frac{y^2}{2}}\]

This demonstrates that the wave functions shown in Figure 2.4 take on the form of the product of the polynomial in \( x \) with a Gaussian function.

The vibrations of any molecular system do not exactly obey the harmonic potential just described. This can be most easily explained for a diatomic system for which the equilibrium position is the equilibrium bond length between the two atoms. When the atoms experience higher vibrational excitation, the potential energy curve must account for the possibility of bond dissociation; hence, the parabolic potential energy function of Figure 2.3 must be substituted by an asymmetric ‘anharmonic’ function shown in Figure 2.5, which includes the cubic term of the Taylor series expansion of the potential energy function.
Table 2.1 The Hermite Polynomials for n = 0 – 4

<table>
<thead>
<tr>
<th>n</th>
<th>$H_n(y)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>$1$</td>
</tr>
<tr>
<td>1</td>
<td>$2y$</td>
</tr>
<tr>
<td>2</td>
<td>$4y^2-2$</td>
</tr>
<tr>
<td>3</td>
<td>$8y^3-12y$</td>
</tr>
<tr>
<td>4</td>
<td>$16y^4-48y^2+12$</td>
</tr>
</tbody>
</table>
Figure 2.4 Vibrational wave functions for the ground state and first two excited states
The ground state wave function is a Gaussian function; excited wave functions are the product of the Gaussian with the Hermite polynomials. Each wave function has a number of nodes as the quantum number and are symmetrical or anti-symmetrical about the equilibrium displacement position.
Figure 2.5 The Morse potential
The Morse potential allows for a more accurate representation of the vibrational motion of a diatomic molecule. This depiction, compared with the harmonic oscillator, takes into account that at high excitation energy the atoms have the ability to dissociate from one another. The dissociation energy can be calculated by subtracting the total well depth of the potential from the zero point energy at \( n = 0 \). In the case of a diatomic system the equilibrium position is the equilibrium bond length, \( d \).
2.3 Instrumentation

The technique used to obtain the IR data discussed in this dissertation was Fourier Transform Infrared Spectroscopy (FTIR). A Fourier transform is a mathematical operation which translates a signal from the frequency domain into the time domain. Presented in this Chapter are the major components of an IR instrument and a how the Fourier transform technique all but eliminated the use of dispersive techniques and made IR data collection more efficient.

2.3.1 IR Sources

Fourier transform instrumentation always uses polychromatic infrared sources. In the instrumentation used for this dissertation, a nichrome source was utilized, heated to 1200 °C. For FTIR studies of biological samples, such as a cell in aqueous media, synchrotron sources have been employed by other groups to boost intensity transmitted through the aqueous medium. A synchrotron accelerates electrons in a circular path by manipulating the forces exerted by the magnetic and electric fields in the path of the particles. Synchrotron radiation occurs when a beam of relativistic electrons is deflected by a magnetic field, and covers a broad spectral range which includes infrared wavelengths. These sources are almost three times brighter than conventional sources and allow sampling apertures to collect near the diffraction limit. Although synchrotron radiation seems ideal for biological samples because of high signal level and the resulting high S/N ratio, it is not a practical solution for clinical or even standard laboratory research since the cost of this source is about 100 million dollars and the circular path of the accelerated electrons typically spans a diameter of several hundreds of meters making it unsuitable, spatially and financially, for the clinical setting.
2.3.2 Michelson Interferometer

Originally, IR data acquisition employed polychromatic radiation emitted from a broadband source after being separated into its monochromatic components in order to obtain any absorbance or transmission spectra. To obtain an absorbance spectrum, Equation 2.12, or a transmission spectrum, Equation 2.13, at every wavelength the ratio is determined of the intensity of the light incident on the sample, $I_0(\lambda)$, and the intensity of the light transmitted by the sample, $I(\lambda)$.\(^1\)

Equation 2.12 Absorbance $A(\lambda) = -\log \frac{I(\lambda)}{I_0(\lambda)}$

Equation 2.13 Transmittance $T = \frac{I(\lambda)}{I_0(\lambda)}$

In the past, the monochromatic components of a polychromatic beam were obtained by the use of a monochromator, Figure 2.6, which employed a diffraction grating to separate the components of light according to Equation 2.14.\(^1\)

Equation 2.14 $d(\sin \alpha + \sin \beta) = n\lambda$

In Equation 2.14, $\alpha$ is the angle of the incident beam, $\beta$ is the angle of diffraction, $n$ is the diffraction order, $d$ is the line density of the grating, and $\lambda$ represents the wavelength of the diffracted light. The grating was rotated to project the desired monochromatic wavelength through the exit slit of the monochromator. The exit slit allowed the sample to be illuminated by one wavelength at a time, which was extremely time intensive.\(^1\)

Nearly all current infrared instruments employ a multiplex interferometric approach in which all wavelengths are sampled simultaneously. The FTIR technique is accomplished with the use of the Michelson interferometer, Figure 2.7. Polychromatic radiation from the source enters the Michelson interferometer as a collimated beam and is redirected onto a beam splitter.
Figure 2.6 Czerny-Turner Monochromator
A monochromator was an integral part of early infrared instruments, employing the use of a rotatable diffraction grating (or prism) to separate polychromatic light from the source into monochromatic components for sample interrogation. The polychromatic light was focused through an aperture, S1, onto a mirror, M1, that directed the light onto the grating, G, to be diffracted. The monochromatic beams were then directed by a second mirror, M2 toward the exit aperture, S2, which would allow one component of the light to exit at a time. The different components were selected by rotation of the grating, G, so that the one component would exit while the rest would be lost inside the walls of the monochromator.
The Michelson Interferometer was the driving force behind the transition from dispersive IR instruments to interferometric methods. The interferometer allows all wavelengths to be sampled simultaneously using a polychromatic source. The IR beam from the source is collimated by a lens (or mirror), L1, and directed onto a beam splitter, B. At the beam splitter, the beam intensity is split so that half is reflected up towards a moveable mirror, M1, capable of moving a distance, x, and the other half at a stationary mirror, M2. The two beams are reflected back at the beam splitter where they recombine constructively or destructively depending on the phase shift induced by the moveable mirror, and then collected at a second lens, L2 to be focused at the detector, D. The phase shift produces an interferogram, which has a distinctly interpretable intensity distribution which can be precisely evaluated because x is known and measured using a reference laser.
by a lens or mirror. At the beam splitter, half of the incident light intensity is reflected by a moveable mirror (capable of displacement along an axis, ‘x’) and the other half of the beam is transmitted onto a stationary mirror. At both mirrors the light is reflected back to the beam splitter where the phase-shifted beam from the moveable mirror recombines constructively or destructively with the beam from the stationary mirror. The light is passed through the sample and is focused onto the detector by an additional lens or mirror. The phase shift caused by the moveable mirror creates an intensity interference pattern referred to as an interferogram which is denoted as J(x). The path difference is determined very accurately via a reference laser, usually a HeNe laser with a much shorter wavelength than the infrared radiation to be analyzed. This laser beam is detected by a monitored and the resulting laser fringe patterns define the precise position of the moveable mirror during data acquisition.¹

In order to obtain an interpretable spectrum from the interferogram, it is necessary to convert the interferogram J(x), from the time (or mirror displacement) domain into the frequency domain, S(ν), by a symmetrical mathematical function referred to as a Fourier transform given by Equation 2.15 and Equation 2.16. These equations imply that the spectrum S(ν) is uniquely defined in terms of the interferogram, and vice versa.¹

Equation 2.15 \[ J(x) = \int_{-\infty}^{\infty} S(\nu) \cos(2\pi \nu x) d\nu \]

Equation 2.16 \[ S(\nu) = \int_{-\infty}^{\infty} J(x) \cos(2\pi \nu x) dx \]

Equation 2.15 and 2.16 are referred to as a Fourier pair. The Fourier transform can be understood from the principles of Fourier analysis, or Fourier series expansion: periodic functions like the interferogram can be expanded by harmonic analysis referred to as the Fourier series.¹⁰ Through a series of substitutions, the complex, periodic function can take on a simpler form and the integral form of the Fourier transform becomes the harmonic analysis with infinitesimally small
frequency intervals. Since the interferogram is sampled at discrete intervals, this process is referred to as a discrete Fourier Transform and is performed computationally by a Fast Fourier transform algorithm, which was developed by Cooley and Tukey in 1965. Early on, this algorithm initially alleviated the issue of large computations, by performing a series of sparse matrix simplifications.

Interferometric methods have three major advantages in comparison with dispersive methods using monochromators. All wavelength intervals are sampled at the detector simultaneously making interferometry much faster, where dispersive methods are limited to one wavelength at a time. Interferometers require no dispersive elements, such as the diffraction grating, which needs to be rotated mechanically. This rotation is associated with a certain error in angle and consequently, in wavelength. In interferometry, this problem is alleviated by using a reference laser to accurately define the position of the moving mirror, which is more accurate than the rotational motion of the diffraction grating. Finally, there are no aperture limiting elements, such as the slit for the monochromator, allowing for a much higher light flux through the spectrometer.

Although the advantages of interferometry have caused the replacement of the dispersive methods in current infrared experimentation, one disadvantage is the need for mathematical procedures such as apodization, which can be understood from Fourier transform theory. The Fourier transform is defined for the infinite interferogram, although it is only collected for a finite number of data points, \( 2^n \). For this reason, to obtain the observed interferogram it is necessary to truncate at a certain value of the mirror travel so the observed interferogram is multiplied by a “box car” function. The spectrum of a truncated data acquisition results in a convolution of the real spectral information with the box car function, which results in a
“convolution” of all spectral bands with the Fourier transform of the function. Shown in Figure 2.8 are some examples of truncation functions, the box car function and another function, and also the line shape functions which they impose upon Fourier transform. It is important to choose a truncation function with a line shape function similar to the normal spectral band shape, for example the second function in Figure 2.8, because the line shape created by a function such as the boxcar function could mask important spectral bands of the sample.

2.3.3 Detectors

For the studies reported in this dissertation, only semiconductor detectors were used. Thermal detectors (which include thermocouples, bolometer and pyroelectric devices) generally are too slow to detect high frequency Fourier components, or are not sufficiently sensitive for the low light levels transmitted by an IR microscope. Semiconductor detectors can be used in either photoconductive or photovoltaic mode. A common photoconductive detector, the one in the FTIR instrument utilized in this dissertation, is the Mercury Cadmium Telluride (MCT) detector. This detector achieves high sensitivity and works on the principle of promoting electrons across the band gap to create a change in resistance. This allows the measurement of the current across the conductivity band. The photovoltaic detector works similarly via promotion of an electron across the band gap to allow the measurement of a potential directly proportional to the intensity of IR radiation. Small detectors are employed for IR instruments because the S/N of the IR spectrum is inversely proportional to the area of the detector, with smaller detectors producing less noise. The typical size for an array detector in an infrared instrument today is between 25 x 25 μm and 250 x 250 μm.
Figure 2.8 Truncation Functions
Illustrated here are two apodization (truncation) functions on the left and their corresponding line shape functions (right). Apodization functions are used to truncate a spectrum so only to illustrate a finite number of sampled wavenumbers for a spectrum. The apodization function is superimposed on the obtained interferogram with the only disadvantage that the function used is superimposed on all bandshapes in the sample spectrum, convoluting the actual sample spectrum. The use of an apodization function similar to the original band shape is necessary, for example the exponential function, because it demonstrates a more Lorentzian line shape function in comparison with the boxcar function which has a corresponding line shape function that is distorted.
2.3.4 Infrared Microscopes

This dissertation exclusively reports microscopically acquired data, since even the largest cells studied (ca. 50 μm in size) are far too small to be studied macroscopically. The infrared microscope design described in this Section is a general design which meets the needs of this dissertation.

The schematic diagram shown in Figure 2.9 is specific to the Perkin Elmer Spotlight 400 (PE 400) Series FTIR microscope for a transmission measurement and shows the general orientation of components in a typical IR microscope. The IR beam from the source, S, is focused from the interferometer onto the sample by a mirror, a Cassegrain condenser or a Schwartzchild objective, C1, all of which operate to minimize the loss of incident radiation. In the case of this instrument, a Cassegrain condenser is utilized. The radiation transmitted by the sample at the stage is collected by a second Cassegrain condenser, C2, and refocused to a remote aperture.10 The sample is located at the position indicated in Figure 2.9 on a computer controlled microscope stage. The stage typically moves in the x and y directions to allow collection of adjacent areas, typically referred to as image mapping.10 A final Cassegrain condenser, C3, refocuses the radiation from the sample onto the detector, D.

The spatial resolution achievable in infrared microscopy is determined by the wavelength and the focusing optics’ numerical aperture (NA). The largest NA for most IR objectives is about 0.6; thus spatial resolution is approximately equal to the wavelength based on Equation 2.17.10

\[
\text{Equation 2.17 Sp. Res} = \frac{61\lambda}{NA}
\]

Thus, the spatial resolution is defined to a first approximation by the wavelength of the light.10
Figure 2.9 Infrared Microscope Schematic Diagram (Transmission)

This schematic diagram depicts the IR optical path for data collection as a transmission measurement. The polychromatic light from the source, S, enters the Michelson interferometer and is focused at the sample from a Cassegrain condenser, C1, (or in some cases a different focusing element). The beam transmits through the sample and emerges on the other side where the light is collected at an additional Cassegrain condenser, C2, (or objective) and finally is focused onto the detector, D, by a third focusing element (C3).
2.3.5 Transmission and Reflectance

Infrared microspectroscopic sampling is conventionally based on the principles of transmission or reflectance, Figure 2.10. Samples are prepared similarly for both transmission and reflectance with respect to dried samples; only the composition of the substrate differs depending on the technique. In the experimentation for this dissertation, samples were not dry, but rather collected as liquid samples in transmission and will be discussed in detail under the experimental Section.

The most commonly used reflective substrates today, specifically for biological IR sampling, are inexpensive low-emissivity (low-e) microscope slides made by Kevley technologies [Chesterland, OH]. The low-e slides consist of a glass microscope slide with a silver coating which is so thin that it is transparent in the visible but reflective in the IR, accommodating visible and spectroscopic observation simultaneously. The silver coating is further protected by a thin coating of SnO2. These substrates are ideal for reflectance measurements as they exhibit no interfering absorption in the spectral range of 4000 to 400 cm⁻¹. Reflectance measurements are easily conducted for dry samples using low-e slides; however, these slides are not suitable for live cell measurements that must be cultured in aqueous environments. The reflective coating becomes compromised when kept in humid conditions or when submerged in aqueous culture medium, producing large spectral band shapes that convolute or distort the sample spectrum, to be discussed in detail in Chapter 3.

Transmission substrates can be used for cultured samples so long as their solubility levels are not high and can remain in culture media for several hours. Typically, substrates used for transmission measurements are ionic compounds which are not polyatomic. Polyatomic ionic compounds are not suitable because their vibrational spectrum will interfere with the vibrational
Figure 2.10 Reflectance and Transmittance Measurements
This illustration demonstrates the manner in which the IR beam interacts at the sample/substrate interface. For reflectance measurements only half of the original source intensity is focused on the sample because half of the focusing optic is used to excite the sample at the stage and the other half of the same focusing optic is used to collect the light for detection. In transmittance the entire incident beam is focused by the entire optic (typically a condenser or objective) and then a second optic is used for the collection of the transmitted beam which has passed through the sample.
absorptions in the spectrally relevant regions of the sample. The refractive index is also an issue for transmission substrates because the higher the refractive index of the material the more light will be lost upon reflection, as shown in Equation 2.18, where $R$ represents reflectivity and $n$ represents the refractive index for the substrate and the sample.

Equation 2.18

$$R = \left( \frac{n_{\text{substrate}}}{n_{\text{sample}}} - 1 \right)^2 \left( \frac{n_{\text{substrate}}}{n_{\text{sample}}} + 1 \right)^2$$

The absorbance of the sample is calculated by the inverse logarithm of transmittance, Equation 2.12 as shown above, and is directly proportional to the molar extinction coefficient, $\varepsilon$, the molar concentration, $C$, and the path length of the sample, $l$, determined by Beer-Lambert Law for absorbance, Equation 2.19.

Equation 2.19

$$A = \varepsilon C l$$

Independent of sampling mode, the characteristic vibrations observed for biological molecules are interpretable and discussed in the subsequent Section.

2.4 Infrared Spectrum of a Eukaryotic Cell

2.4.1 Vibrational Modes of Biological Molecules

Eukaryotic cells are primarily composed of protein, RNA, DNA, carbohydrates and phospholipids, the individual cellular components responsible for the intricate cell processes and functions. Cells demonstrate a very specific infrared spectrum that is a superposition of the individual cellular components; shown in Figure 2.11 are the spectral bands that make up that superposition. Monitoring the vibrational frequencies and band shape changes for a cellular
Figure 2.11 IR Spectra of Cellular Components
This series of spectra represent the cellular components which are superimposed to represent the IR spectrum of a eukaryotic cell. Starting at the bottom in orange are the vibrations specific to phospholipids; red represents the DNA vibrational bands; blue represents RNA specific bands; and light green and dark green spectra represent two different proteins with different secondary structure.12
spectrum have been used for evaluations regarding secondary structure, protein hydration, and
disease state, among several other biological events, as far back as the mid-1900s.\textsuperscript{10}

The normal modes of vibration for infrared active molecules are classified as stretching,
bending, torsions, out of plane bending (twisting, wagging), and in plane bending (rocking,
scissoring). A nonlinear molecule with \(N\) atoms exhibits \(3N-6\) normal modes of vibration\textsuperscript{9}. The
stretching normal modes involve the symmetric or asymmetric motion of two or more atoms
bonded to a central atom as shown in Figure 2.12. The motion of the bending normal vibration is
characterized by a change in the angle between two bonds involving the same atom.

Although proteins and peptides are composed of the 20 different amino acids, the nature
of most peptide bonds is the same, Figure 2.13. The amide linkage of the peptide bond exhibits
characteristic vibrations upon excitation described as the amide A, the amide I (carbonyl stretch
mode), the amide II (C-N stretch mode), and the amide III (N-H/C-H deformation mode), Table
2.2. These spectral frequencies, along with many others intrinsically belonging to biological
molecules, absorb in the mid-IR (MIR) range between 4000-400 cm\(^{-1}\). Also, shown in Figure
2.14 are characteristic vibrations of the phospho-diester linkage found in DNA, RNA and
phospholipids. Vibrational spectroscopy usually reports the abscissa of a spectral plot in units of
inverse wavelength or wavenumber (cm\(^{-1}\)) , \(\bar{\nu} = \frac{1}{\lambda}\) because it is directly proportional to the
energy, \(E = \hbar c \bar{\nu}\) and the frequency.

2.4.2 Vibrations Specific to Liquid Water

Water is a strongly polar molecule and in its liquid form it exhibits strong vibrational
frequency bands in the same region of important spectral information regarding cellular
components. The three normal modes of liquid H\(_2\)O appear near 3750 cm\(^{-1}\), 3650 cm\(^{-1}\) and 1620
Figure 2.12 Normal Modes of Vibration
Example of normal modes of vibration. Any molecule exhibits continuous atomic motion, which is a manifestation of the energy content of matter. These motions can be described in terms of the normal modes of vibrations. When one of these vibrational coordinates is excited, a photon of the appropriate energy is absorbed, and the amplitude of the vibrational mode increases. Vibrational modes are (somewhat arbitrarily) classified as bond stretching, angle deformation, wagging and out-of-plane deformation modes.
Figure 2.13 Peptide Bond (Amide Linkage)
All peptide bonds are formed by an amide linkage joining one amino acid to the other. This amide linkage is responsible for the very characteristic vibrational modes referred to as the amide I, the amide II, the amide III and a few other modes. These normal vibrational modes are a result of the motion of the atoms within the amide linkage. The Amide I vibration is mostly the stretching motion of the carbonyl group, the Amide II is caused by the stretching motion of the CN bond and the deformation of the CNH group, and the Amide III is caused by the deformation of the hydrogen atoms on the nitrogen atom and the adjacent carbon atom of the second amino acid.
Table 2.2 Spectral Assignments
Listed are several wavenumber assignments representative of specific bond vibrations inherent to cellular components.14

<table>
<thead>
<tr>
<th>Absorption Peak (cm(^{-1}))</th>
<th>Assignment</th>
<th>Cellular Constituent</th>
</tr>
</thead>
<tbody>
<tr>
<td>3290</td>
<td>Amide A (N - H Stretch)</td>
<td>Protein</td>
</tr>
<tr>
<td>3050</td>
<td>Amide B (N' - H Bending, 1st Overtone)</td>
<td>Protein</td>
</tr>
<tr>
<td>3010</td>
<td>Olefinic (C - H Stretch)</td>
<td>Lipid</td>
</tr>
<tr>
<td>2960 - 2930</td>
<td>CH(_3) (Asymmetric Stretch)</td>
<td>Lipid, Protein</td>
</tr>
<tr>
<td>2925 - 2920</td>
<td>CH(_2) (Asymmetric Stretch)</td>
<td>Lipid, Protein</td>
</tr>
<tr>
<td>2874 - 2870</td>
<td>CH(_3) (Symmetric Stretch)</td>
<td>Lipid, Protein</td>
</tr>
<tr>
<td>2855 - 2850</td>
<td>CH(_2) (Symmetric Stretch)</td>
<td>Lipid, Protein</td>
</tr>
<tr>
<td>1735</td>
<td>Ester (C = O Stretch)</td>
<td>Lipid</td>
</tr>
<tr>
<td>1717</td>
<td>Purine (C = O Stretch)</td>
<td>Nucleic Acid</td>
</tr>
<tr>
<td>1666</td>
<td>Pyrimidine (C = O Stretch)</td>
<td>Nucleic Acid</td>
</tr>
<tr>
<td>1655 - 1650</td>
<td>Amide I (C = O Stretch)</td>
<td>Protein (α-Helical Secondary Structure)</td>
</tr>
<tr>
<td>1640 - 1630</td>
<td>Amide I (C = O Stretch)</td>
<td>Protein (β-Sheet Secondary Structure)</td>
</tr>
<tr>
<td>1580</td>
<td>COO(^-) (Asymmetric Stretch)</td>
<td>Protein</td>
</tr>
<tr>
<td>1560 - 1500</td>
<td>Amide II (N - H Bending)</td>
<td>Protein</td>
</tr>
<tr>
<td>1470 - 1405</td>
<td>CH(_2) (Symmetric and Asymmetric Bending)</td>
<td>Protein, Lipid</td>
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<td>1400</td>
<td>COO(^-) (Symmetric Stretch)</td>
<td>Protein</td>
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<td>1380 - 1250</td>
<td>CH(_3) (Symmetric and Asymmetric Bending)</td>
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<td>1280</td>
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<td>Protein</td>
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<td>Protein</td>
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<tr>
<td>1204</td>
<td>Amide III of Collagen</td>
<td>Protein</td>
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<td>Glycogen (C = O Stretch)</td>
<td>Carbohydrate</td>
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<td>1028 - 1020</td>
<td>Glycogen (C - O - H Deformation)</td>
<td>Carbohydrate</td>
</tr>
<tr>
<td>1080</td>
<td>PO(^2-) (Symmetric Stretch)</td>
<td>Nucleic Acid, Lipid</td>
</tr>
</tbody>
</table>
Figure 2.14 Phosphate Vibrations
Demonstrated here is part of the phosphate backbone of DNA that is partially responsible for symmetric and anti-symmetric phosphate vibrations which contribute to the observed IR spectrum of a eukaryotic cell. RNA and phospholipids exhibit similar vibrations.
cm⁻¹, the anti-symmetric stretching mode, symmetric stretching mode and deformation mode (bend), respectively are shown in Figure 2.15. The deformation mode of liquid H₂O is the vibration that poses the biggest obstacle to biological studies using FTIR. The broad deformation band at 1620 cm⁻¹ is superimposed on the strong amide I position of cellular proteins, and masks both the amide I and amide II bands. When subtracting the liquid water background, care has to be taken not to equalize the intensities of the Amide I and Amide II bands, a process which will be referred as “overcompensation” later in this dissertation. The abnormal band profile imposed by liquid water on the pertinent spectral region of cells is the main reason experiments performed on live samples are avoided. In order for cellular samples to be kept viable during FTIR experimentation it is necessary to keep cells in aqueous culture medium during data collection. Only in recent years has the FTIR community begun to investigate the possibilities for performing live IR spectroscopic measurements and methodologies for negating the conflicts presented when working in aqueous environments.¹³,¹⁴ This dissertation presents work completed towards advancing the field of FTIR microspectroscopy for medical diagnosis by performing experiments on live individual HeLa cells leading the field to develop methods for personalized cancer treatment in the future.
Figure 2.15 Normal Modes of Water
This Figure illustrates the three normal modes of vibration that are specific to water in its aqueous form. The mode denoted with a box specifically interferes with the wavenumber region representative of proteins and is the cause of the abnormal band profile described in the text.
2.5 References


Chapter 3: Experimental Design and Methods

3.1 Introduction

Chapter 3 serves as a detailed account of the experimental design for all work performed in this dissertation and the instrumental parameters used for acquisition of data. The majority of live FTIR spectroscopic measurements collected in this dissertation which were subjected to analysis were acquired using the PE 400 IR microscope. Another FTIR instrument, the Smiths Detections IlluminatIR instrument (Smiths Detection, Warrington, UK), was also utilized, but only to test the initial designs of the live cell sample chamber described in this Chapter. This Chapter also includes a detailed account describing the optimization of data collection made possible with the use of MATLAB [Mathworks, Natick, MA] based pre-processing algorithms. Subsequent Section 3.3 lists the components specific to the PE 400 (as each was described previously in Chapter 2) and provides an explanation for how the two different collection modes, point mode and image mapping, were used for the purposes of this research. Following data acquisition, samples were pre-processed one of two ways using MATLAB based algorithms, both described in this Chapter, and all data were smoothed and normalized uniformly prior to chemometric analysis.

3.2 Sample Preparation

The human cervical cancer cell line, HeLa [ATCC, Mannassas, VA], was chosen for the research presented in this dissertation because of its well-known reputation of being easy to culture (often referred to as a ‘lab weed’) and because it is an adherent cell line and can be seeded onto IR substrates. Presented in this Section are the culture conditions describing how samples for data acquisition were prepared, the obstacles presented during the initial
experimental stages regarding substrate incompatibility to aqueous conditions and the development of a live cell sample chamber designed to accommodate live cells in the PE 400 IR microscope during data acquisition.

### 3.2.1 Cell Culture Conditions

All samples were cultured HeLa cells and were initiated from culture frozen and stored at -80 °C in the laboratory or obtained (frozen) directly from the distributor (ATCC, Manassas, VA). Cells were initially cultured in 75 cm³ culture flasks [Corning, Lowell, MA] and incubated at 37 °C and 5% CO₂. Stored samples were typically frozen in culture medium supplemented with 10% dimethyl sulfoxide (DMSO). DMSO protects the cell membrane when frozen, assuring viability when thawed. Cultures were allowed to progress for one complete cell cycle (ca. 24 hours) and then culture medium was exchanged to eliminate DMSO from the cells and their culture medium. Although DMSO is necessary to retain viability of frozen cultures, it can present toxic effects, and it was necessary to eliminate any potential factors that may interfere with the cells during experimentation. HeLa cells were cultured to confluence before samples were prepared for data acquisition.

Upon reaching confluence, HeLa cells were trypsinized by commercially available 0.25% trypsin/2.2 mM EDTA solution [ATCC, Manassas, VA]. After cells were detached from the culture flask, trypsinization was halted by addition of 5 mL cultured medium supplemented with 10% fetal bovine serum (FBS). Cells were centrifuged to obtain a cell pellet and the supernatant was discarded. The cell pellet was resuspended via pipette aspiration in fresh culture medium supplemented with 10% FBS, the volume of which was determined based on the amount of cells recovered (smaller re-suspension volumes for smaller cell pellets). Cells were then cultured
directly onto substrate. For initial stages of this research, cells were cultured onto reflectance substrates (“low-e” MirrIR slides [Kevley Technologies, Chesterland, OH]). For all time course and drug studies reported in this dissertation, cells were cultured on transmission substrates (CaF$_2$ windows [International Crystal Laboratories, Garfield, NJ]). Substrates were placed in Petri dishes and submerged in culture medium supplemented with 10% FBS before cells were transferred by pipette to the center of the substrate and placed in the incubator to adhere. The rectangular low-e slides measured 76 mm x 25 mm x 2.25 mm for preliminary experiments and 76 mm x 40 mm x 2.25 mm for later experiments to accommodate the flow of culture medium (it was necessary to adjust the width of the slides because of the size of the CaF$_2$ top window). The low-e slides were submerged in 13 mm x 13 mm sterilized square disposable polystyrene Petri dishes with approximately 15 mL of culture medium supplemented with 10% FBS for incubation.

The round CaF$_2$ windows, employed after the transition from reflectance measurements to transmission measurements, measured 32 mm x 3 mm and were submerged in 47 mm culture dishes [Millipore, Billerica, MA] with approximately 5 - 7 mL of culture medium supplemented with 10% FBS.

Samples were incubated for approximately 24 hours after cells were seeded onto substrates, and then the culture medium was exchanged to eliminate any remaining trypsin/EDTA solution. Initial experiments employed the low-e reflective slides; however, a broad spectral band appeared in the low wavenumber spectral cutoff region. It became apparent that these substrates were not ideal and that substrates accommodating transmission measurements were more robust for live cell experiments. This is discussed in more detail in the subsequent Section 3.2.2 including a description of the adjustments applied to the experimental
procedure as a result of the reflective substrate incompatibility in the aqueous cell culture medium.

3.2.2 Reflective Substrate Incompatibility

Initial experimental procedures utilized low-e slides for two main reasons other than their reflectivity in the IR: they were extremely inexpensive (approximately $1.00 per slide) and after experimentation samples were easily discarded. Early experiments were performed with individual HeLa cells cultured directly onto the low-e substrates, so each slide remained in culture medium for a minimum of 24 hours and a maximum of two days. Low-e slides were an ideal reflective substrate for IR microspectroscopy because they are completely reflective to IR light in the spectral region of 4000 - 400 cm\(^{-1}\) due to a layer of Ag on the glass slide, while remaining transparent to visible light. The slide transparency to visible light permitted visible image capture during IR data acquisition and the cell morphology could be monitored simultaneously to monitoring the IR detectable biochemical composition. Low-e slides presented as a favorable substrate for clinical cellular samples and have been used in much of the research in the Laboratory at Northeastern University\(^2\)-\(^6\), however, they were not useful for cultured samples because the reflective coating became compromised in aqueous conditions. A broad spectral band in the low wavenumber region of each spectrum, near the spectral cutoff, was observed and is illustrated in Figure 3.1. The confounding band was attributed to glass, which would be detected once the reflective surface is compromised. The intensity of the broad band was variable for the individual spectra collected near the cutoff region and introduced concern about the substrate. As previously mentioned, this was addressed by implementation of transmission allowable substrates, specifically round CaF\(_2\) windows. Early experimentation to
Figure 3.1 Substrate Incompatibility
Demonstrated is an infrared spectrum of a cell cultured onto a low-e slide. A broad spectral band appeared in the low wavenumber region and varied in intensity from sample to sample. Occasionally, the broad band appeared with negative absorbance intensity. Later, this absorption band was attributed to glass vibrations, which resulted from inconsistencies in the reflective coating after extended time in aqueous culture medium.
test the initial design of the sample chamber to see if IR throughput could be achieved through
the sample was done using an IR instrument available to the lab, the Smiths Detections
IlluminatIR FTIR microscope. This instrument was not ideal for live cell experiments and could
only be used for reflectance measurements, described later in this Chapter. As the experiments
progressed the PE 400 was utilized. The broad band in the low wavenumber region confirmed its
origins to be due to glass, and not an artifact of the instrument. The intensity of the band would
varied between positive and negative values dependent on whether the area was being observed
in the background spectrum, appearing negative after the ratio, or in the sample cell location,
appearing positive after the ratio. A detailed explanation of this data acquisition is in subsequent
Section 3.3.

The broad spectral band had not been observed previously in the laboratory and to my
knowledge, was not reported previously in the literature. Most likely it was not observed because
most of the samples in the laboratory were clinical samples mounted to low-e slides by
cytocentrifugation. Cytocentrifugation utilizes minimal solution, typically a buffer solution, to
suspend exfoliated cells. This sample procedure for exfoliated cells utilizes a Cytospin (Thermo,
Waltham, MA) to spin deposit 1-3 drops of cell suspension by centrifugal force onto slides. The
funnels used for the spin deposit procedure employ an absorbent filter layer to eliminate the
solution upon deposition of the cells. The sample area of the slide remains in-contact with the
solution for seconds, not enough time to disrupt the reflective layer. The reflective layer of the
low-e slides exhibits a visible green tint. The visible green tint of the reflective layer decomposed
the longer the slides were submerged in water, revealing the glass beneath. The areas where the
glass appeared through the reflective layer were the areas with the strongest intensity interfering
in the low wavenumber region.
The first few live cell chamber designs were planned to accommodate low-e slides, described in detail in Section 3.2.3. Once it was confirmed that samples could not be cultured on low-e slides, CaF$_2$ windows were utilized instead. The windows were specially ordered to fit the final live cell chamber design, described in the subsequent Section. The dimensions for each round window were 32 mm x 3 mm with two drilled holes, separated by a distance of 18 mm, to accommodate the flow of culture medium. A schematic drawing of the specially ordered CaF$_2$ windows is demonstrated in Figure 3.2. CaF$_2$ is only slightly soluble in water and eliminated the risk of observing glass absorptions in the low wavenumber region, as well as the concern of introducing solubilized silver atoms into the culture medium, which could potentially create a toxic environment for the cells. Detailed descriptions and depictions of the live cell chamber through the stages of the design are included in the subsequent Section 3.2.3.

3.2.3 Live Cell Sample Chamber Design

Prior to any experimentation, it was necessary to develop an initial design for a sample chamber that would be easily adapted for obtaining spectra of live cells. The initial design was to allow data collection of cells that were immediately removed from the incubator and transferred to the instrument in the absence of culture medium flow. It was important and necessary to keep the cells isolated inside the chamber in solution and prevent any solution from leaking into the instrument. Achieving proper isolation of the sample cells also eliminated the potential for introducing bacteria into the sample area, which could potentially induce cell death. Figure 3.3 represents the initial design for the sample chamber. Since the initial goal was to obtain usable spectra in aqueous conditions, the chamber was not designed for long term data collection. A
Figure 3.2 CaF₂ Window Designed for the Live Cell Sample Chamber
The schematic drawing indicates the dimensions for a specially designed CaF₂ window. Two holes were drilled, each 9 mm from the center position, to accommodate the flow of culture medium that was channeled through the live cell sample chamber. This window was specifically ordered at the dimensions indicated in the Figure to accommodate the design of the sample chamber.
Figure 3.3 Initial Sample Chamber Design
Demonstrated are the schematic drawings and dimensions for the initial design of the live cell sample chamber. The design depicted above was mostly intended to serve as a base for a design and excluded any plans for maintaining temperature control or culture medium flow. Depicted in (A) was the original plan for the bottom base plate, completely constructed from aluminum. Aluminum was the material of choice because of its conductive properties, which would make temperature control possible in future designs. At the center of the bottom base plate was a hole for visible illumination. This original design included four screws that penetrated the base plate from the top down so that the aluminum top plate, (B), could be placed on top and then twisted into place to hold a CaF₂ window on top of the low-e slide with a Teflon spacer in-between. The top plate depicted in (B) was replaced by a new design, (C), constructed from Plexiglass and the screws were rearranged to enter from the bottom up. The top plate shown in (C) would then just slide down on top of the upright screws and pressure was applied by four nuts which held the top plate securely in place on top of the sample area.
base was developed with no method for delivering fresh culture medium and no way to control the temperature.

In the initial chamber design, cells were cultured on a low-e slide and the slide placed in an aluminum block, referred to as a base plate, shown in Figure 3.3 (A). The original base plate design had a rectangular inset to fit a standard 76 mm x 25 mm x 2.25 mm sized low-e slide. A Teflon spacer, (Harrick Scientific, Pleasantville, NY) 12 µm thick, was used between the low-e slide and a round CaF₂ top window (diameter 25 mm, thickness 2 mm), to create a space for the cells to remain intact in solution. The thickness of a HeLa cell adhered to a substrate and not undergoing apoptosis is approximately 8 µm. The window and spacer were held in place by a top plate, Figure 3.3 (B and C). Originally, the top plate, Figure 3.3 (B), fit over four screws which entered from the top of the base plate to be twisted clockwise into position. The top plate was designed and constructed from Plexiglass, Figure 3.3 (C). The four screws were moved to penetrate the cell chamber from the bottom up, and the top plate depicted in Figure 3.3 (C) slipped over the four screws to be held in place and four nuts were placed to apply pressure. Replacing the aluminum top plate with a Plexiglass top plate allowed the design to fit more securely, and the Plexiglass allowed better visibility both during setup and also in the instrument. The ability to see the edges of the top window and the Teflon spacer through the top window allowed better visualization of potential leaks, which sometimes formed around the spacer due to insufficient pressure. Also, occasionally a wrinkle in the Teflon spacer would occur creating a channel for the solution at the sample spot to escape.

Data were collected successfully using the initial live cell sample chamber on a Smiths Detections IlluminatIR microscope. An in depth discussion of this instrument will not be included because data were not used for analysis, only to test the actual chamber. The
IlluminateIR FTIR instrument was used to collect individual cell spectra using an all-reflecting objective (ARO) to test the physical practicality of the sample chamber design and the ability to detect the IR beam throughpath. The IlluminatIR FTIR instrument was not practical for future long term live cell experiments because there was no setup to securely purge the instrument and stage area with dry air (-40°C dew point) to eliminate atmospheric water vapor spectral contributions. In an attempt to eliminate as much water vapor as possible, a dry air hose was mounted onto the stage at the sample and a second dry air hose was aimed into the nose piece of the microscope. This crude attempt to purge the instrument was sufficient for testing the initial chamber design. A major concern was whether the path length created by the 12 μm spacer between the slide and the CaF2 window would be too large, causing prohibitive extinction of the IR beam by the liquid. Although the commercially obtained spacer was reported to be 12 μm, when the bottom and top plates were tightened and the path length was probed spectroscopically, the path length was calculated to be approximately 8 - 10 μm with pressure applied. This calculation was performed using a fringe pattern spectrum obtained due to the sample area being empty of everything except air. Interference at the window air interface caused a sinusoidal pattern referred to as a fringe pattern. Using the fringe pattern allowed an accurate determination of the path length to be calculated based on Equation 3.1, where \( b \) is the path length in millimeters and \( N \) is the number of fringes in the spectral region between \( \nu_1 \) and \( \nu_2 \), the start and end wavenumbers of the chosen spectral region.\(^7\)

\[
\text{Equation 3.1} \quad b = \frac{10N}{2(\nu_1 - \nu_2)}
\]

Results were encouraging; Figure 3.4 illustrates a spectrum of an individual HeLa cell cultured on a low-e slide that was contained between the slide and a CaF2 window with buffered saline solution (BSS) as aqueous medium. This spectrum was collected using the initial sample
Results using the initial live cell chamber design were encouraging. The spectrum in this illustration is of one individual HeLa cell in buffer solution. Observed here are the spectral bands representative of a cell that demonstrated that useful spectral data could be obtained under these conditions. The abnormal band profile caused by the aqueous contribution was addressed in later experiments. The only major issues presented were the strong rotational-vibrational bands associated with water vapor, superimposed at the maxima of the amide I positions and the broad band, appearing negative in absorbance, near the low wavenumber region at ca. 1400 cm\(^{-1}\). The water vapor was not a concern because this would be addressed in the transition from the IlluminatIR instrument to the PE 400 infrared microscope, which was equipped with a proper dry air purge. The broad negative band at 1400 cm\(^{-1}\) was due to the degenerative substrate used and led to the change from reflectance measurements to transmission measurements.
chamber design shown in Figure 3.3. Although the signals observed were strong and relatively free of noise, the presence of the strong rotational-vibrational transitions of atmospheric water vapor, superimposed on the amide I positions, demonstrated the necessity of a proper purging system. The PE 400 IR microscope accommodated dry air purge (-40 °C dew point) by providing a purge box around the microscope sample area (Figure 3.5), and a good purge system of the microscope and the optical bench. However, the fact that the signature spectral bands of the cell were observable was important and confirmed that this approach was feasible. A sufficient throughput for collection was achieved providing observation of nearly 0.1 absorbance units at the amide I position, despite the strong absorption in the low wavenumber region as a result of the reflective slide incompatibility with aqueous water.

The next stage in the sample chamber development introduced the ability to flow culture medium across the sample location and created a means for maintaining the temperature. The bottom aluminum stage plate was redesigned with an inlet and an outlet protruding from opposite sides for introduction of fresh culture medium with a simultaneous exit of waste culture medium; this is illustrated in the schematic Figure 3.6 (A). Aluminum was used because of its heat conductive properties. Two heating cartridges with a 6 mm diameter and 55 mm length (Vulcan Electric, Porter, ME) were housed in the aluminum bottom plate. The heating cartridges were maintained at 37 °C by an external power supply (Circuit Specialists Inc., Meza, AZ) capable of 0 – 18 volts and 0 – 2 amps. The size of the top plate, Figure 3.6 (B), was adjusted to match the newly designed bottom plate, but the overall design was maintained.

At this point, the top plate still consisted of a thin Plexiglass plate to hold the sample together with the Teflon spacer in-between, the low-e slide and the CaF₂ cover, Figure 3.6 (B). Also, it was necessary to adjust the design to accommodate the working distance of the PE 400
Figure 3.5 PE 400 IR Microscope with Dry Air Purge
Shown in the photograph is the PE 400 IR microscope during data collection. The purge box, indicated with the yellow arrow, completely isolates the sample stage area of the microscope and is constantly purged with dry air (-40 °C dew point). The instrument is also purged inside the microscope and inside the optical bench.
Figure 3.6 Second Live Cell Chamber Design
The schematic depicted above represents the adjustments made to the initial live cell chamber design to accommodate the CaF$_2$ substrate. In this second design, the bottom plate (A) includes a channel for culture medium to flow to the sample cells and houses two heating cartridges to maintain the chamber temperature at 37 °C. The top plate (B) is still made out of Plexiglass and screws down at the four corners to apply pressure and keep the sample windows together.
microscope, which was used in all subsequent experiments. Adjusting the sample chamber width dimensions was not sufficient to accommodate the working distance; also, the issue presented by the reflective substrate had not been resolved at that time. It was not until after this design that experiments officially transitioned to transmission measurements. Since experiments were still conducted in reflectance collection mode, the lower Cassegrain below the stage of the PE 400 IR microscope was removed to create additional room for the stage to be lowered and accommodate the working distance. The bottom and top plates were also made as thin as possible in an attempt to assist in the accommodation of the working distance.

The design including the heating cartridges and the channel for the culture medium flow, both housed in the bottom plate, meant that the bottom plate had to be taller than the original design measuring 12 mm high. Also, the Teflon spacer was a major flaw with the second design as the spacer was not compatible with the flow-through system. The spacer was extremely flexible; thus, when pressure was applied and culture medium was driven into the chamber, the spacer would always slip and cause leakage, even if the two plates were pressed together very tightly. The leaks due to the spacer and the incompatibility of the low-e slides with aqueous solution prompted the transition from low-e slides to CaF₂ windows; an attempt was made at a new approach to accommodate the working distance. Transmission data collection requires that the Cassegrain be in place for data collection. Demonstrated in Chapter 2, Figure 2.12 shows a schematic representation of the beam path for the PE 400 IR microscope. S represents the infrared source which was focused onto the Michelson Interferometer. Indicated with by C1, C2, C3 are the Cassegrain focusing elements and D represents the detector. The width of the bottom plate including the heating cartridges and flow channel put the focal plane of the sample higher than the working distance of the PE 400 IR microscope. The working distance was addressed by
implementing two solutions. (1) The first was the design of a stage plate to be exchanged for the original stage plate screwed into the microscope, shown in Figure 3.7. This replacement stage plate was designed using Plexiglass to reduce heat transfer from the sample chamber to the microscope and was built to allow the sample chamber to sit slightly lower in the microscope stage than it would with the original stage plate. (2) The second solution was to make the bottom plate thinner so that the focal plane was at a lower point on the stage. This was accomplished by removing the heating cartridges from the bottom plate and housing them in the top plate, which now had to be constructed out of aluminum as well. The flow channel remained in the bottom plate.

The only issue that remained was to create a cavity for the cells so they were kept intact and viable. It was necessary to replace the Teflon spacer with something that could be properly sealed with applied pressure. It was important to address this to ensure the live sample chamber would not leak upon flow of the culture medium and to keep the sample area from drying out. In an attempt to keep the concepts of the latest design of the live cell chamber the same, the issue was resolved by exchanging the Teflon spacer and the 25 mm diameter CaF₂ top window with a CaF₂ window shown in Figure 3.8 that had an inset well etched out (Biotools, Jupiter, FL). The inset well eliminated the need for a Teflon spacer and created a strong seal around the sample area with a path length of approximately 10 µm.

The changes made with the substrate and the sample chamber were the final adjustments in the design, illustrated in Figure 3.9. This depiction demonstrates all the working components of the live sample chamber together as it appeared when set up for data collection. Shown in Figure 3.8, the culture medium held in a reservoir (A), was directed to the sample using flexible Tygon tubing (Cole Parmer, Vernon Hills, IL) mimicking an intravenous line. The flow rate of
Figure 3.7 Plexiglass Stage Plate
This illustration shows the design of the Plexiglass stage plate made in part to accommodate the working distance of the PE 400 with the live sample chamber. The four corners of the sample chamber were able to sit slightly lower than the plane of the stage. The stage plate was constructed from Plexiglass in order to reduce heat transfer from the live sample chamber to the instrument and was made to exactly match the dimensions of the original stage plate of the instrument to easily be exchanged.
Figure 3.8 Biotools CaF$_2$ Window
A CaF$_2$ window with an inset well etched out was used to create a cavity for the cells adhered to the drilled CaF$_2$ window (Figure 3.2) to remain intact and viable. This window was used to replace the need for a Teflon spacer. The surface of the well window in contact with the drilled window created an efficient seal preventing leaks with appropriate pressure applied from the top plate.
Figure 3.9 Final Sample Chamber, Complete Assembly
Illustrated is the complete assembly of the live cell sample chamber used in the collection of all live data for this dissertation. The culture medium was held in a reservoir (A) and delivered by a gravity driven flow controlled via a thumb regulator (B). Culture medium was driven across the sample area entering through the inlet (C) and exiting at the outlet (D). Temperature was controlled to remain a constant 37 °C by two heating cartridges (E) housed on either side of the CaF$_2$ windows at the center. Pressure was applied to the top plate at four points where the two plates screwed together to keep the sample area sealed.\textsuperscript{8}
the culture medium was controlled using a thumb regulator (B). The inlet and outlet for the
culture medium channel remained in the bottom aluminum plate, (C) and (D) respectively, and
the heating cartridges (E) remained housed in the aluminum top plate. The cross sectional view
in Figure 3.10 shows the 10 µm path length cavity created by the Biotools CaF2 window
allowing the cells to remain intact and viable for experimentation. The final design, shown in the
photograph Figure 3.11, both the bottom (C) and top (B) plates were constructed out of
aluminum, which allowed the temperature to be maintained at a constant 37 °C at the sample.
The Plexiglass stage plate designed to exactly fit the sample chamber (A) and was the same
dimensions as the original stage plate. The temperature was constantly monitored using the
Fisher Scientific Traceable Dual Thermometer (Thermo Fisher Scientific, Cambridge, MA) by
attaching the dual probes to the edge of the CaF2 window near the sample spot. The probes were
kept in contact with the window the entire collection time and monitored constantly.

3.3 Data Acquisition

The PE 400 IR microscope is capable of data collection via two methods: point mode
data collection and spectral mapping; both are discussed in this Section and demonstrated in
Figure 3.12. Preliminary research conducted during the development of the live sample chamber
and during the investigation for practicality of performing FTIR measurements in an aqueous
environment employed point mode data collection. Data collection was optimized after the
development of the preprocessing routine referred to as noise adjusted principal component (NA-
PC) reconstruction. With the implementation of the NA-PC algorithm, collection methods were
adapted and spectral image mapping was employed for the remaining research in this
dissertation.8,9 Discussed in this Section is a detailed explanation of point mode collection and
**Figure 3.10** Cross Section of Sample/Substrate Area In the Sample Chamber
The cross sectional view depicted above demonstrates how the two CaF$_2$ windows, the one with the holes drilled for culture medium flow (Figure 3.2) and the well window (Figure 3.7), came together to produce a cavity with a pathlength of 10 µm for the cells to remain intact and viable.
Figure 3.11 Photographs of The Final Sample Chamber and Stage Plate
Shown in this photograph is the final design for the Plexiglass stage plate (A) tailored specifically to be exchanged for the original stage plate of the PE 400 IR microscope to accommodate the working distance with the sample chamber. Also shown are the bottom plate (B) and the top plate (C) which make up the live cell sample chamber, both are open at the center to allow visible image capture and IR transmission measurements and both are completely constructed out of aluminum for easy maintenance of a constant 37 °C temperature.\textsuperscript{8}
Figure 3.12 Point Mode vs. Image Mode Data Acquisition

Demonstrated above (A) represents the visible image capture of the sample which is used in (B) point mode data acquisition to set up aperture boundaries around every individual cell and (C) image mode data acquisition for defining an area to be imaged at every pixel. The point mode collection method acquires a spectrum for every individual cell whereas the image mode collection method collects an image map of every cell so that every pixel represents spectral information.¹¹

(Figure image courtesy of Dr. Max Diem)
spectral image mapping, expanding on the concept which was discussed in a publication in the *Journal of Biophotonics* by Schubert, J. M., *et al* (2010).10

### 3.3.1 Point Mode Collection

Point mode data collection can be thought of as a ‘point and shoot’ based methodology for acquisition. For this collection mode, spectra are collected using a 100 µm single element HgCdTe detector. A 50 µm² aperture was used to limit the field of illumination specific to the sample area size of a typical HeLa cell. Point mode data collection is demonstrated in Figure 3.12 where the visible image captured (A) of the sample area is used to set up aperture boundaries (B) in a point by point manner to obtain one spectrum at every individual cell. The 50 µm² aperture was used for all point mode data collection. It was necessary to collect a background spectrum near the sample cell for an accurate ratio of sample and background single beam spectra to obtain a spectrum that was a true representation of the observed cell. The background single beam spectrum for all live cell experiments was taken of aqueous culture medium adjacent to the cell. This necessitated collection of a background spectrum adjacent to every individual cell to anticipate any variations in cell chamber thickness (path length) from spot to spot. This was a time-consuming proposition, since collection of the background requires equal or more time than collection of the cell single beam spectrum. The spectra were independently saved as single beam files rather than obtaining the ratio them immediately. The reason for saving independent single beam spectra for each cell and each background was to be able to externally apply an aqueous water correction at a later time.

All point mode data collection parameters were kept uniform for all experiments employing this collection mode. All spectra were collected 128 interferograms at every
background position and 128 interferograms at every cell location, with 4 cm\(^{-1}\) spectral resolution. The major disadvantage to this methodology was that it was time consuming and manually laborious. The time required to collect a background position and a sample position for one cell was approximately 6 minutes at these parameters. That time does not include the to locate each cell and align the microscope aperture. The time constraint was particularly an issue for time course experiments, as it was necessary to relocate the same individual cells at various time points quickly in order to acquire an accurately representative study of cellular changes over time. Conversely, spectral image mapping involved less manual labor and eventually allowed faster collection of cellular data.

### 3.3.2 Image Mapping

Spectral image mapping employed the 8 x 2 detector element HgCdTe focal plane array of the PE 400 infrared microscope to raster scan a designated image area, as shown in Figure 3.12 (C). The live cell experiments collected as image maps for this dissertation were all 1 mm\(^2\) areas collected at 6.25 \(\mu\)m x 6.25 \(\mu\)m pixel size. Prior to collecting the image, a single beam background spectrum for all 16 detector elements was collected, by co-adding 120 interferograms in an area void of cells. Images were collected at 8 scans per pixel and 8 cm\(^{-1}\) spectral resolution. Originally, data were collected at 4 cm\(^{-1}\) resolution, the same as for the point mode data collection; however, using 8 cm\(^{-1}\) resolution saved a factor of 2 for collection time.

The actual spatial resolution of the PE 400 IR microscope was 12 \(\mu\)m\(^2\) as demonstrated in the literature using military targets for calibration.\(^{10}\) Image definition was enhanced because of oversampling (although the spatial resolution remained 12 \(\mu\)m\(^2\)) where the microscope stage was stepped by the pixel size of 6.25 \(\mu\)m, effectively oversampling by a factor of 2 at every step.
illustrated in Figure 3.13. Oversampling does not improve spatial resolution, but improved image definition, which has been previously demonstrated using synchrotron radiation sources in the literature.\textsuperscript{11} Image collection is an automated process where the only manual involvement occurred during setup of the image marker to dictate the area to be sampled, which included selecting the area to be imaged on the stage, entering the collection parameters and focusing the image marker over the sample area.

In the literature, spectral imaging was used for tissue samples and not cellular samples, because cells are dispersed on the window, and there is a lot of unoccupied background space in-between. The amount of background space for a spectral image map is significantly more than the amount of space occupied by the cells, and the time it takes to collect an imaged area in comparison with using point mode collection appears to be a less favorable choice. In the subsequent Section is an introduction to the preprocessing and analysis methodologies used in this dissertation. Provided in this dissertation is an explanation for why spectral imaging was a better choice. Spectral image mapping at 8 cm\textsuperscript{-1} spectral resolution in conjunction with the preprocessing algorithms, provided an optimized methodology for live cell experimentation with a stride in the right direction for personalized cancer treatment plan development by allowing collection of more quality data in less time.

3.4 Data Processing and Analysis

The data processing and analysis conducted for this dissertation were all completed using MATLAB based algorithms developed in the laboratory. Since the data collection methodology for this dissertation transitioned from point mode collection to spectral imaging as discussed in the previous Sections of this Chapter, it was necessary to pre-process the data \textit{via} two
Figure 3.13 Example of Oversampling to Improve Image Definition

The actual spatial resolution for the PE 400 IR microscope was determined, using resolution targets, to be 12 µm at 1000 cm⁻¹. Images are raster scanned from left to right, indicated by the black boxes and the green arrow on top. The image definition is increased by spectral oversampling. The microscope stage step distance was 6.25 µm indicated by the shaded pink box. This figure illustrates that at every step, the overlapped region is recollected, effectively oversampling the image area at every step.
independent protocols. All point mode data acquired were stored by the PE 400 software as Perkin-Elmer, Inc. specific files (.sp files) for every individual cell background and cell spectrum collected, whereas all image maps were stored as .fsm files containing 25,600 spectra. Due to the difference in how the data files were stored, all point mode data were exported and saved in MATLAB format before the ratio of each background and the cell spectrum was performed based on Equation 2.12, previously discussed in Chapter 2. All image maps stored as .fsm files were imported directly into a preprocessing algorithm referred to as NA-PC and then analyzed using a second algorithm, PapMap\textsuperscript{10}, to acquire a spectral database, each discussed thoroughly in the Subsequent Sections 3.4.2 and 3.4.3 respectively. Files stored as .fsm files were already ratioed, and contained transmission data.

The data processing methodologies after the preprocessing step(s) converged, as all data were normalized and smoothed in the same manner. Spectral variance was demonstrated using chemometric analysis, specifically Principal Component Analysis (PCA)\textsuperscript{12}, discussed in the subsequent Section 3.4.4. In the earliest stage of this research, an investigation was carried out to determine whether liquid water compensation of the observed spectra was necessary. To this end, a MATLAB-based water correction algorithm was developed under the guidance of Dr. Miloš Miljković. This algorithm was used to eliminate the liquid water contribution in the spectrum of a cell by minimizing the water combination band located near 2100 cm\textsuperscript{-1} and is discussed in detail in the subsequent Section 3.4.1.\textsuperscript{13}

3.4.1 Water Correction Algorithm

The abnormal band profile demonstrated in Figure 1.3 (Chapter 1) is a result of the difference in the water volume at the sample in comparison with the water volume of the
background location. The cell targeted for acquisition occupied most or all of the cavity path length created between the two windows. The area where the background was collected was occupied by the liquid medium surrounding the cells and filling the cavity, shown in Figure 3.14. Thus, when ratioing the cell and the background, the background spectrum overcompensated the actual cellular spectrum, resulting in a reduced amide I intensity, and distorted amide I/amide II intensity ratios.13

In order to establish whether a liquid water correction was necessary for analysis it was necessary to develop an algorithm that would allow the option to output both corrected and uncorrected spectra from the raw data for comparison. It would be beneficial to have a program capable of both because depending on what the final decision was, the algorithm would be prepared for immediate data processing. Illustrated in Figure 3.15 is the graphical user interface (GUI) that was developed. The GUI was operated by manually scaling the water contribution to minimize the water combination band near 2100 cm⁻¹ to produce a flat baseline in that region. This procedure had been suggested previously in the literature.14-17 The intensity of the water combination band was adjusted by the application of a scaling factor to the background single beam spectrum of Equation 2.12 (Chapter 2) to produce Equation 3.1 where \( \text{rawcell} \) was the single beam spectrum for the cell, \( \text{bkgd} \) was the single beam spectrum for the background and \( \text{SF}1 \) was the scaling factor applied to produce CellAbs, the observed absorbance spectrum for the cell being acquired.13

\[
\text{Equation 3.1 } \text{CellAbs} = -\log(\text{rawcell}) + (\text{SF}1 \times \log(\text{bkgd}))
\]

However, this procedure, while correcting the distorted amide I/amide II intensity ratios, produced distortions in the low frequency (ca. 1000 – 1150 cm⁻¹). This distortion was found to result from the transmission cut-off of CaF₂. Since the single beam spectrum of the aqueous
**Figure 3.14** Pathlength Difference For a Position Occupied by a Cell and the Background Position

This depiction demonstrates a closer look at a cell occupying the cavity created by the two CaF$_2$ substrates. The beam penetrating an area with a cell experienced less liquid environment than an area unoccupied, indicated by the black arrows. The difference in aqueous environment is the cause of the abnormal band profile observed in cellular spectra.
Figure 3.15 Liquid Water Correction Graphical User Interface

The graphical user interface (GUI) demonstrated above allowed manipulation of scaling factors that affected the liquid contribution of the culture medium, as well as a CaF\(_2\) contribution to offset the distortion introduced in the cutoff region upon compensation of the liquid component. The scaling factors are manually adjusted using the sliding bars to the right of the spectrum. The top spectrum represents the spectrum as the adjustments were made and the bottom spectrum shows the original spectrum. Presented in the analysis tool box on the bottom right of the Figure is the option to cut the spectral region of interest. The user is able to calculate the second derivative of the spectrum, perform Savitzky – Golay smoothing algorithm and vector normalize prior to saving the spectrum.
medium also contained the intensity variations transmitted by the CaF₂ windows, a scaling factor $SF₂$, to scale a spectrum of a CaF₂ was introduced. The scaling factor $SF₂$ was proportional and opposite to the first scaling factor and was introduced to the ratio for cell absorbance in Equation 3.2.\textsuperscript{13}

Equation 3.2 \[ CellAbs = -\log(rawcell) + (SF₁ \times \log(bkgd)) + (SF₂ \times \log(CaF₂)) \]

The algorithm, whose GUI is shown in Figure 3.15, operates by first importing the single beam spectrum for the cell, the background and the CaF₂ individually into the program. At this point the unscaled single beam ratio of cell and background spectra was displayed, without correction or analysis, in the white boxes. The larger of the two boxes showed the adjustments to the spectra in real time as they were being applied. The smaller of the two boxes remained the same and showed the original raw spectrum. The water and the CaF₂ scaling factors were each applied with the two slider bars shown on right hand side of Figure 3.15. Depicted below the slider bars was the analysis tools menu. The first white box was used to indicate the spectral region of interest and the region was then applied to the raw spectrum via the ‘Spectral Cut’ button directly below. The button ‘Perform ratio’ permanently applied the scaling factors dictated by the sliding bars. The blank window below ‘Perform Ratio’ required three inputs for the number of smoothing points, polynomial order and derivative. The smoothing points were applied using the Savitzky-Golay\textsuperscript{18} smoothing algorithm, polynomial order was always set to 2 for the purposes of this work and finally the derivative order was set to 0 for the absorbance spectrum and 2 for the second derivative. These parameters were applied by the push button directly below, and finally the last push button performed vector normalization.

This approach effectively compensated for the CaF₂ and water background contributions, and produced spectra with a normal band profile when the water combination band at 2100 cm\textsuperscript{-1}
was minimized. However, we found – in spite of obtaining much more normal-looking spectra – that this correction was mostly cosmetic and did not affect the further analysis of the data, for the following reasons. All data analysis is commonly conducted on the second derivative of spectra because bandwidths are reduced and spectral discrimination is increased and background offsets and slopes disappear. Discrete shoulders of bands and frequency shifts are harder to detect in the original absorbance data, but more readily observed in the second derivatives. Thus, individual cell spectra were smoothed and derivatized using a Savitzky - Golay moving window algorithm and vector normalized. Figure 3.16 (A) illustrates an individual cell spectrum both before and after aqueous water correction and (B) the second derivative spectrum for each plotted together for comparison. After comparing a number of corrected and uncorrected 2nd derivative spectra (Figure 3.16), it was concluded that the water correction was unnecessary because in spite of an absorbance intensity change at the amide I position, the spectral band positions remained unchanged. The intensity change at the amide I position was expected because the maximum of the water spectrum used for the correction lies nearby. At this point the decision was made to conduct all data analysis for this dissertation without applying a water correction.

3.4.2 Data Analysis for Image Data: Noise Adjusted Principal Component (NA-PC) Reconstruction

All .fsm image files, storing 25,600 spectra from each 1 mm x 1 mm imaged area, were subjected to the NA-PC reconstruction algorithm before final analysis. The NA-PC reconstruction algorithm was used to improve the signal-to-noise ratio at each individual pixel of the image. The use of this algorithm introduced the notion that image acquisition, although
Figure 3.16 Individual Cell Spectrum Before and After Water Correction

Illustrated in (A) an individual cell spectrum before and after water correction using the GUI. (B) Illustrates the second derivative of the absorbance spectra shown in (A) plotted together for comparison. The conclusions were made that water correction was unnecessary because the spectral frequencies of the bands were unaffected by correction. The major observation was the intensity change at the amide I position, which was expected upon subtraction of the water contribution. Based on these conclusions, the decision was made to perform analysis on all uncorrected spectra.
collecting data from areas not occupied by cells, was not wasting time and made it a more practical solution for fast and efficient collection of quality live cell data.19

Image files were imported into the NA-PC reconstruction algorithm and baseline offset. This baseline correction subtracts the lowest intensity point of each spectral vector. Subsequently, the spectral region from 1626 - 1676 cm$^{-1}$ (the amide I band position associated with cellular proteins) was integrated to produce a raw intensity map of the imaged area. Using the raw intensity map, discrimination was made between the areas that were individual cells and the areas that were void of cellular matter, only consisting of liquid water background. The areas void of cellular material were used to construct a “noise correlation matrix” from which the eigenvector matrix would be extracted and stored for the next computation, further discussed in appendix II. 19

Similarly, the pixel areas shown to contain cellular material were also used to create a raw intensity map and a “signal covariance matrix”. Figure 3.17 illustrates regions of noise only (A) and regions of cellular material (the signal covariance matrix) (B). These matrices were diagonalized separately to provide eigenvector matrices. The signal eigenvector matrix was re-sorted using the noise eigenvectors and individual pixel spectra were reconstructed after noise adjustment from the first 30 loading vectors of the PCA. The first 30 loading vectors, also referred to as principal components (PCs), were chosen because 30 presented the ability to reconstruct the data while ensuring that all the spectral components were still included, minimizing the noise. Most of the spectral contributions are contained in the first 10 PCs. PCA is discussed in greater detail in subsequent Section 3.4.4; however, it is important to note that the early loading vectors tend to represent the actual spectral components. The chosen 30 PCs allow a safe margin to assure that no spectral data were discarded. Also, by using the first 30 PCs the
The illustration above represents the ‘noise-only’ areas of the image for the noise reduction afforded by NA-PC reconstruction, Panel A. The area represents only background, including substrate and liquid water contributions. Shown directly below Panel A is the spectrum of one pixel from the noise matrix. Panel B illustrates the areas containing cellular material. Directly below is the pixel spectrum from a cell. This spectrum is shown prior to noise adjustment and is a raw pixel absorbance spectrum.
Spectral noise is reduced by a factor of 3. This can be equated to performing a 9 point Savitzky–Golay smoothing window function to the data, which is common practice to reduce spectral noise and is discussed in detail in subsequent Section 3.4.4. NA-PC reconstruction presents the advantage that rather than applying a uniform smoothing function over the entire spectral region, the noise at every individual wavenumber is being considered. Essentially, this program presented the ability to construct a noise specific response to the noise profile specific to the instrument used for data acquisition. 19

At this point, the spectral images contain all the cellular spectra broken up by pixel. Every individual cell was composed of approximately 10 – 90 pixels. An additional MATLAB based algorithm, PapMap10, was used for the co-addition of these pixels corresponding to an individual cell to create an average absorbance spectrum for each cell.

3.4.3 Data Analysis for Image Data: PapMap

PapMap is an algorithm developed in the laboratory in the course of research aimed at automatic spectral cytopathology10. It was used in this dissertation to combine the pixel spectra representative of each individual cell, after NA-PC reconstruction. The combination of pixels for each of the cells would produce a database of cellular spectra for analysis, where each spectrum represented the averaged pixel spectra for each individual cell. 10 The algorithm recognizes which pixels make up an individual cell, and performs an averaging of these pixels, subject to certain constraints, to producing an output of one spectrum per cell.

In order to ascertain which pixel spectra belong to a cell, the algorithm presents the user with a binary mask that indicates areas occupied by cells, as shown in Figure 3.18. 20 The user can select a maximum number of pixels, usually between 10 and 90, to constitute a cell. Larger
Figure 3.18 Binary Mask (PapMap)
Illustrated is the visible image (A) of the spectrally imaged area and the corresponding binary mask (B) created using the PapMap algorithm. In the binary mask all areas representing cellular material are shown in white and the background areas void of cells are shown in black. The blue arrow indicates the ability to dictate cellular size in PapMap to exclude anything not representative of an individual cell, for example a cluster of cells. This algorithm is used to co-add the pixels representative of each individual cell to obtain one averaged spectrum for each cell.\(^2\)
areas in the binary mask are due to clumps of cells or debris. In Figure 3.18, a cell cluster indicated by the blue arrow is eliminated from the binary mask (B) because of the cell size limits. Further criteria are used when co-adding pixel spectra to eliminate pixels of low S/N, and edge artifacts. These aspects have been discussed in the literature. This was particularly useful for live cell experiments because the goal was to obtain spectra of individual cells. The samples were all cultured samples, and as cells divided they tended to remain close to one another. Although most samples were cultured no longer than 24 hours to eliminate clusters, occasionally cell congregation was unavoidable and those clusters could be eliminated at this stage of data processing.

Upon completion of the pixel co-addition, each raw spectrum was stored in a database. The database correlated to the stage coordinates of the cell with a name tag was also constructed and saved to be used at a later time; if needed, the data could be revisited and maps of the same sample area could be compared with a visible image captured during data acquisition. This was particularly useful for re-examining the same live cells over time for any morphological changes that may have occurred. At this stage, data had only been pre-processed and were prepared for spectral smoothing and normalization prior to PCA.

### 3.4.4 Chemometric Analysis

The final analysis for all data presented in this dissertation was an unsupervised multivariate chemometric method known as Principal Component Analysis (PCA) to demonstrate whether or not the dataset contained correlated variance. PCA reduces the dimensionality of a data set from multidimensional space and projects the data using fewer dimensions. PCA extracts the spectral components from the data set based on variance,
illustrated in Figure 3.19 where (A) represents the original data (in this case shown, an artificial data set). (B) represents the basis set obtained from the covariance matrix eigenvectors, where the first eigenvector spectrum represents the mean of the data set, the second the largest variance among the data, the third the next largest variance and so on, and (C) represents the reconstructed data set based on the weighted variance components. In this example data set, the variance is located at the low wavenumber region where the low intensity band exhibits a frequency shift, and the high wavenumber region where there is a relative intensity change between the two most intense peaks. None of these changes are detectable by visual inspection of the spectra. The reconstructed data set shown in (B) is based on a linear combination of the three PCs, where the scores indicate the contribution of each PC to the reconstructed spectra. When these scores are plotted against each other for each individual cell, a “scores plot” shown in Figure 3.20 is obtained. The data that exhibited no spectral variance appeared as a random ‘shotgun blast’, an example shown in Figure 3.20, where all the data points appeared scattered with no real pattern. Data that indicate a spectral change, even very small changes, are demonstrated as a split between data points, an example is shown in Figure 3.21 where the cells indicated with blue demonstrate variance in comparison with the cells indicated in red. PCA was a useful technique in the analysis of live individual cells because of its extreme sensitivity, which enabled detection of small spectral variance that may have been missed upon visual observation of spectra.
Figure 3.19 Principal Component Analysis (PCA)

Principal component analysis is a multivariate analytical technique useful for plotting variance among large datasets because it reduces the dimensionality of the data set from multi-dimensional space and projects the data using fewer dimensions. Variance is determined from the original spectra (A) and used to create a basis set (B) and then the data is re-expressed as a linear combination and plotted based on the weighted components (PCs) where the early PCs represent spectral variance and the later PCs represent instrument noise.\(^5,^{13}\)

(Figure image courtesy of Dr. Max Diem)
Figure 3.20 Scores Plot Indicating No Spectral Variance
Illustrated above is an example data set which exhibits no detected spectral variance. A scores plot showing no variance presents as a ‘shotgun’ blast where there is no obvious trend among the data points. Every data point represents one individual cell.\textsuperscript{13}
Figure 3.21 Scores Plot Indicating Spectral Variance
Illustrated is an example data set demonstrating strong detected spectral variance. Data which exhibit variance cluster away from one another as shown in this depiction. The variance for this example data set was shown along the first two PC’s and from this plot it can be determined that the cells indicated with the blue circles are spectrally distinguishable from the cells indicated with red circles. The spectral variance could be evaluated by inspecting the eigenvectors of the those two axes.22
3.5 References


7. *Calculating the Pathlength of Liquid Cells by FTIR*; Pike Technologies Spectroscopic Creativity.


Chapter 4: Monitoring Induced Spectral Changes in Live Cells under Aqueous Conditions

Parts of this Chapter have been published and are included in Appendix I


4.1 Introduction

Live cell FTIR studies allow for the real time detection of biochemical changes in individual cells as a result of different chemical and environmental perturbations and may provide the means for predicting how diseased cells would respond to potential drug treatments. There are several ways a methodology such as this could be used, for example to pre-screen for drugs that show no effect on cells or to elucidate the response time of a drug before it takes action on diseased cells. Ultimately, a tool such as this could lead to better prognoses and fewer long-term side effects for patients.

After the completion of the live cell sample chamber design, the focus of this research was aimed at testing the chamber and demonstrating the ability to detect induced spectral changes in the cervical cancer cell line, HeLa, in response to perturbations in their cellular environment. The results presented in this Chapter illustrate the ability to manipulate conditions that would invoke cellular response(s) and the analytical sensitivity necessary to detect changes that occurred on the sub-cellular level.¹
4.2 Review of Experimental Procedures

The experimental conditions and analyses for the data presented in this Chapter were described in detail in Chapter 3. Data were collected utilizing the point mode methodology employing a 50 µm² aperture (field of view). Each cellular spectrum was acquired as a ratio of a single beam spectrum of the cell, with the aperture set to straddle each cell, and a single beam background spectrum collected from an equal area adjacent to every cell. After ratioing, the second derivative was calculated, the data were Savitzky-Golay smoothed and vector-normalized prior to PCA. The data presented in this Chapter illustrate some of the first results obtained during the initial experimental period of this research. Despite the obstacles presented by liquid water, these were among the first spectral data in the literature to be obtained reflecting a long-term study of individual cells in aqueous medium. The other significant attribute of these results was that all these data were obtained using a commercially available source rather than a synchrotron beam source used for most of the live cell studies shown in the literature.²⁻⁹

4.3 The Ability to Monitor Live Individual HeLa Cells Using FTIR

In an attempt to observe induced spectral changes in live HeLa cells using the live cell sample chamber, it was first necessary to collect data reflecting live cells under normal culture conditions to ascertain cellular viability. In Figure 4.1, the data represent the mean spectra for 13 individual HeLa cells monitored over the course of five time points. The 13 cells were scanned consecutively, and the time points indicated in the legend reflect the time that cell number 1 was scanned (or re-scanned for the consecutive time points). These data were corrected for the water contribution in order to obtain the more ‘normal’ looking band profile, discussed previously in Chapter 3, Section 3.3.1. These results were extremely encouraging for cells in aqueous
Figure 4.1 Mean Absorbance Spectra for HeLa Cells Kept Under Normal Culture Conditions

Demonstrated in this Figure are the mean absorbance spectra for thirteen individual HeLa cells over five time points monitored consecutively using FTIR microspectroscopy. Cells were kept under normal culture conditions including culture medium supplemented with FBS kept at a constant 37 °C. These absorbance data were corrected for aqueous water contributions as described in Chapter 3. Visual inspection of the absorbance spectra suggests that there is no variance observed between the cell spectra as was expected since these cells were continuously maintained under normal culture conditions to promote cellular viability.
conditions for several reasons: (1) the first observation for these data was reproducibility over the experimental time points. The reproducibility was the first indication that no spectral changes were occurring; this was further verified for second derivative spectra (Figure 4.2) and subsequent PCA analysis. (2) The second significant observation was that most of the signature spectral bands were present, except for clear discrimination in the low wavenumber cutoff region. The degradation of S/N below 1100 cm\(^{-1}\) is due to two factors: the transmission cutoff of CaF\(_2\), and the detector sensitivity, which was further degraded by the fact that the sample area detected was 50 x 50 µm and the single element detector measured 100 x 100 µm. Since the small sample area was projected onto the larger detector element, the entire detector area was not being illuminated and contributed to the noise. Single element detectors are larger than array detectors and so they have a worse detectivity. This will be discussed further in Chapter 5 during the comparison of improved S/N of the low wavenumber region when collecting spectral image maps. The important illustration made in Figures 4.1 and 4.2 was that the water pathlength was not preventing FTIR measurements of live cells, and these data demonstrate good S/N despite the confounding factors of this approach.

The second derivative spectra showed better discrimination of the spectral components in the mean spectra. Each time point shown in the legend was the same as in Figure 4.1, where the time indicated reflects the point at which cell number 1 was scanned (or re-scanned). The only significant spectral variance is observed in Figure 4.2 between the amide I and the amide II peaks; this region is confounded by contributions of the water vapor vib-rotational spectrum. The lack of spectral variance can be better illustrated using the PCA scores plot shown in Figure 4.3 where every individual data point reflects one individual cell spectrum.\(^1\) The scores plot, plotted over PC 1 and 2, demonstrate no discernible pattern indicating a lack of spectral variance. The
Figure 4.2 Mean Second Derivative Spectra for HeLa Cells Kept Under Normal Culture Conditions
The second derivative for the mean spectra of the 13 individual HeLa cells over five consecutive time points, the absorbance spectra were shown Figure 4.1. The second derivative of the data demonstrated better spectral discrimination and illustrated the similarity of the mean at each time point.¹
Figure 4.3 PCA Scores Plot for HeLa Cells Kept Under Normal Culture Conditions

PCA was performed on second derivative data to produce the scores plot demonstrated above. The scores plot demonstrates a lack of spectral variance over the five consecutive time points in which 13 individual cells were monitored via FTIR microspectroscopy. These results were encouraging because the sample chamber was kept under normal culture conditions to promote cellular viability, and these results suggested that this goal was achieved. ¹
analysis for these data produced four significant loading vectors (eigenvectors) and although only 1 and 2 are used to demonstrate the data in Figure 4.3, all four were plotted and inspected, and no spectral variance was observed. These results were encouraging as this demonstrated that over the course of an 11 hour experiment, cells could be maintained under normal culture conditions using the live cell sample chamber with no induced spectral variance. The next challenge in this research was to perform an experiment using the same sample volume and time points but under conditions predicted to induce a spectral change, for example induced death. This lead to the results provided in the subsequent Section 4.3.1.1

**4.3.1 Detected Changes as a Result of Cellular Starvation**

The initial results obtained suggested the ability to monitor individual cells using the live cell sample chamber following the same individual cells over the course of 11 hours under normal culture conditions with no detected spectral variance. Cells exhibit extreme sensitivity to their environment and vibrational spectroscopy can be used to detect biochemical changes occurring at the cellular level. In an effort to confirm the results just described in the previous Section, and simultaneously show the ability to observe spectral changes in individual cells, experiments were conducted to induce spectral changes in individual cells by perturbing the cellular environment *via* conditions known to induce cellular death.

The experimental procedure involved induction of apoptosis *via* cellular starvation. This was achieved by exchange of the constant flow of the normal culture medium for PBS. The experimental design was developed to complement the previous experiment shown in Figures 4.1 - 4.3, where data of 13 individual HeLa cells were collected at five time points for comparison purposes. The culture medium was exchanged for PBS as soon as the cells were
transferred to the live cell sample chamber from the instrument, so the starvation began at the start of data collection. Figure 4.4 demonstrates the mean second derivative spectra for the 13 individual HeLa cells monitored consecutively over 5 time points.\(^1\) The spectral changes observed are subtle yet detectable, where the change appears mostly in the amide region. The subtle transition of the spectra over time became more apparent in the scores plot shown in Figure 4.5, where the initial time point is shown in blue and the transition occurred gradually over the course of the 11 hour experimentation time.\(^1\) As the starvation progressed there was a transition among the cells represented by the final two time points where they began to separate along PC2. It appears as though after the 270 minute time point the change began to take place.\(^1\)

Figure 4.6 represents second derivative data of an experiment performed on a different date with same nutrient deprivation conditions described for Figures 4.4 and 4.5 using fewer time points over nearly 3 hours monitoring 8 individual HeLa cells. The conclusions were similar, observing an induced spectral change as a result of cellular starvation \textit{via} PBS but with fewer data points the spectral variance is more distinctly observable. Shown in Figure 4.6 are the processed mean second derivative spectra of the three time points. The spectral regions were enlarged in the top panel to point out that no spectral changes were occurring in the \(-CH\) stretching vibrational region near 3000 cm\(^{-1}\), but the spectral variance was detected at the amide vibrations representative of proteins. The scores plot shown in Figure 4.7, using the processed data shown in Figure 4.6, illustrated a similar subtle change as in the longer cellular starvation experiment illustrated in Figure 4.5.

These results described above were extremely exciting because they demonstrated that spectral changes could be observed using the custom built live cell sample chamber along with FTIR microspectroscopy. The variance was subtle, but this was expected because apoptosis is
**Figure 4.4** Mean Second Derivative Spectra of HeLa Cells Starved *via* PBS

The second derivative spectra illustrated in this figure represent the mean spectra of 13 individual HeLa cells scanned consecutively over the course of 5 time points in PBS solution in an effort to mimic the experiment under normal conditions (Figures 4.1-4.3). However, the conditions in this experiment were manipulated to demonstrate detectable spectral changes induced through deprivation of normal culture medium. Most of the changes observed occur around the amide I and II positions, 1650 cm⁻¹ and 1550 cm⁻¹ respectively. The changes are subtle, yet detectable.¹
PCA was performed on HeLa cells deprived of culture medium by exchange of normal culture medium supplemented with FBS for PBS in an experiment similar to that shown in Figures 4.1-4.3 demonstrating HeLa cells under normal culture conditions. In the data illustrated by this scores plot, an induced spectral change is observed when cells were deprived of the nutrients necessary to maintain cellular viability. The variance is best visualized by focusing on the first time point, indicated in blue, and the final time point, displayed in purple. For comparison purposes the same plot with only those two time points is shown to the right.
Figure 4.6 Mean Spectra for Nutrient Deprived Cells Over a Shorter Experimental Duration

Demonstrated in these spectra are the mean second derivative analyzed data for cells deprived of normal culture medium supplemented with FBS. Culture medium was exchanged for PBS in an attempt to induce early stages of apoptosis and mimic previous results demonstrated in Figures 4.4 and 4.5. This study represented a shorter study using fewer data points but the overall results were similar. The reason for using fewer data points was so the transition is more easily observed.
Figure 4.7 PCA Scores Plot of Nutrient Deprived Cells Over A Short Experimental Duration
HeLa cells were kept under conditions to promote induced apoptosis through nutrient deprivation with exchange of normal culture medium for PBS. This experiment demonstrates a similar subtle change in cells over time in comparison with the same study performed over a longer experimental time shown in Figure 4.5. Fewer time points were used to more clearly discriminate the variance. The reason the trend appears in the opposite direction as the previous starvation PCA scores plot is that the variance occurred in different PCs and the direction of the trend (in this case right to left) is independent of the variance.
not a fast or a drastic cellular death process like necrosis. Contrary to necrosis, apoptosis is a gradual, ordered series of events which is consistent with the observed subtle shift demonstrated in Figures 4.5 and 4.7 and in the changes of the spectral features near the amide I and II positions as indicated in Figure 4.4. To correlate the FTIR results demonstrated in these Figures, visual images of the cells were captured to demonstrate the simultaneous morphological changes, also suggestive of apoptosis. Illustrated in Figure 4.8 is an example of the visible images captured for one of the individual cells included in the data analysis of the nutrient deprived cells for the longer starvation experiment (11 hours). The red arrow points to the cell observed and discussed. The initial morphology of the cell, demonstrated in Panel A, was typical for cultured adherent cells, with distinct pseudopods reaching away from the cell’s center. This morphology is typical for viable adherent cultured cells, however, as the cells were starved the morphology began to change. The most drastic observations are shown in D and E, which indicate that the cell had become more round and the appearance of the membrane surface was no longer smooth, indicative of apoptosis.

These experiments demonstrated the successful implementation of the live cell sample chamber with the ability to monitor induced spectral changes, resulting as one of the longest FTIR experiments performed on live cells. As this was a significant accomplishment, 11 hours was still a short experimental time for reflecting cellular changes and did not encompass the duration of an entire cell cycle, which typically occurs over 24 hours. In order to apply this technique and yield a more amenable tool for pre-screening purposes of potential drug treatments, it was necessary to demonstrate the ability to monitor individual cells for significantly longer experimental times, ideally 24 hours because changes in cell composition upon drug treatment vary in the time required for interaction with cells. In some cases, the
Figure 4.8 Images of An Individual HeLa Cell Deprived of Culture Medium
Demonstrated in this Figure are the visible images captured for one of 13 individual cells (the red arrow is indicating the top cell of the two depicted in each panel) monitored consecutively over five time points while deprived of normal culture medium. The experiment demonstrated induced spectral changes using FTIR microspectroscopy. Illustrated here are observed morphological changes that occurred as a result of cellular starvation. These changes correlate with the gradual changes observed in the PCA scores plot and mean second derivative spectra shown in Figures 4.5 and 4.4, respectively. Also these changes were indicative of apoptosis, characterized by loss of membrane extension and over all round and less smooth characteristics of the cell membrane.1
interaction occurs on a slower time scale and the ability to perform longer experimental times could prove beneficial.

### 4.3.2 Acquiring Data of Live Cells Over Extended Experimental Periods

The next set of experiments were designed to include measurements over a longer time course to assure that the sample chamber allowed sufficient time to observe potential long term spectral changes. These experiments were to monitor the same individual HeLa cells over the course of 24+ hours. A limit of 20 individual cells were monitored spectrally and captured visually; this limit was dictated by the collection rate limit of over 6 minutes per cell acquisition. The cell number was set to 20 individual cells so the duration of each individual time point would not be excessive, exhibiting a more manageable representation of detected spectral changes. Figure 4.9 illustrates the PCA scores plot of the data monitoring 20 individual cells over 24+ hours where no discernible spectral variance was observed. Seen in the legend there are two times listed for each time point. The first time reflects the time that cell number 1 was scanned for that collection period. The second time reflects the time that the last cell, number 20, was scanned during the collection period. Cells were kept under normal culture conditions with a constant flow of fresh culture medium supplemented with FBS, and the sample was maintained at a constant 37 °C. There were no spectral changes observed in the spectra.

Prior to this experiment, it was hypothesized that the cells might divide, and stages of the cell cycle could be observed for live cells. Prior efforts from this laboratory had reported spectral changes with the cell cycle when cells cultured were synchronized and spectra were collected for dried cells at various time points.$^2$
Figure 4.9 Individual Live HeLa Cells Monitored Over 24 Hours

HeLa cells were kept under normal culture conditions for 24+ hours using the live cell sample chamber. The lack of spectral variance shown in the scores plot indicates that the cells remained viable for the duration of the experiment. This was significant because it confirmed that spectral changes observed *via* cellular starvation and other experiments perturbing the environment of the cells were not a result of stress imposed on the cells from the sample chamber itself. Also these results were significant because they represent the longest study of individual cells using FTIR.
For the observation time of this experiment, 24+ hours, one should expect to see the morphological changes of a few dividing cells, since the HeLa cells were not synchronized; thus, some cells should have been at the various stages of the cell cycle at the commencement of the experiment. However, the cells remained as they were and may have entered a quiescent phase due to their environmental conditions, such as space restrictions in the cell chamber. This experiment represents the longest FTIR microspectroscopic study performed to follow individual live cells in the literature.

4.3.3 Observing Drug Induced Spectral Response

The experiment described in this Section represents the first attempts to monitor drug induced spectral changes in live HeLa cells using the live cell sample chamber and the PE 400 instrument. Cyclophosphamide monohydrate is an antineoplastic agent; its modes of action and mechanism for how it targets cells will be thoroughly discussed in Chapter 5, Section 5.5. The use of this drug in the experiment presented here was strictly preliminary to ascertain the ability to detect drug-induced changes. Figure 4.10 demonstrates the PCA scores plot illustrating data collected of cyclophosphamide monohydrate treated cells on four separate dates, indicated in blue, plotted with cells kept under normal conditions acquired on five different dates, indicated in red (more dates included to compare the same relative number of cells). Results were extremely encouraging as there were noticeable differences observed in the clustering of the individual cells represented by the red data points in comparison with the blue shown in Figure 4.10.

An important observation in these data was that the spectra shown in blue clustered closely together in comparison to the spectra shown in red, which is an intuitive observation.
The data points in blue represent individual live cells exposed to (ca. 300 μM) cyclophosphamide monohydrate, which were acquired on 4 different dates. The data points in red represent cells collected over 5 different dates (to have equal number of spectra for comparison), kept under normal culture conditions. The spectral region analyzed was a shorter wavenumber region than typically used, 1500-1200 cm⁻¹, to exclude significant noise in the cutoff region and variations in the water contributions caused by pathlength differences between different dates of collection.

The significant observation made was despite the shorter spectral region used for analysis, the data demonstrated a distinct variance with respect to cells exposed to cyclophosphamide monohydrate, which clustered more tightly together in comparison with cells kept under normal conditions, which exhibited a greater variable spread. This preliminary drug result demonstrates the ability to observe spectrally induced changes as a result of chemotherapeutic drug exposure.
because it would be more likely that the drug treated cells would all respond similarly upon the introduction of an agent that disrupts the normal cellular processes. The cluster representing the normal (control) cultured cells, shown in red, exhibits a larger spread that slightly overlaps with the cyclophosphamide monohydrate treated cells, shown in blue. This is also intuitive because the spectra of cyclophosphamide monohydrate exposed cells were acquired prior to cell death, indicated by cells still adhered to the substrate, which may account for the overlap. Interestingly, the cells represented by blue squares and circles were incubated with the drug for 24 hours and the cells represented by the blue triangles and diamonds were only incubated for 12-18 hours. This may account for the spread of blue data points, specifically the blue triangles and some of the blue diamonds, which appear further into the red clustered symbols on the right side of the scores plot in Figure 4.10.

These data were preliminary, and the spectral region observed for analysis of all data compared in Figure 4.10 was 1500-1200 cm\(^{-1}\). The reason that such a restricted wavenumber region was utilized was because the point mode aperture based methodology employed the 100 \(\mu m^2\) single element detector and insufficient spectral quality was obtained in the low wavenumber region near the CaF\(_2\) cutoff. This may account for the poor signal at the low wavenumber regions because by using a 50 \(\mu m\) aperture the entire detector element was not utilized. Smaller detectors are ideal for IR measurements because of the higher detectivity. This is further discussed in Chapter 5. The phosphate symmetric stretching vibration at 1060 cm\(^{-1}\) was not consistently observed making any confident interpretations using that region difficult. Furthermore, several background locations were collected, specifically one for every cell collected. This introduced variability due to changes in the path length volume occupied by aqueous culture medium, so a confident conclusion could not be made in spectral changes.
observed over the amide I position either. The use of such a small wavenumber region contributed to the observation that the variance shown in Figure 4.10 was not substantially differentiated; however, the significance in this experiment was that regardless of these confounding experimental obstacles, it was still possible to detect drug induced spectral changes. The challenge that arose from these data was to develop an optimized methodology to allow more data to be collected with improved spectral quality. The detailed experimental approach to attain this goal is addressed in the following Chapter.

4.4 Summary of Preliminary Live Cell FTIR Microspectroscopy Results

The results represented in this Chapter were all acquired using the initial point mode aperture based methodology of the PE 400 FTIR microscope. These early experiments, using this methodology and the live cell sample chamber designed to accommodate the PE 400 FTIR microscope, demonstrated that monitoring individual live HeLa cells in aqueous culture conditions for extended experimental times was possible. This approach was utilized to successfully monitor induced spectral changes occurring in individual cells as a direct result of perturbations to the environment to which the cells were exposed. These experiments included a cellular starvation experiment depriving cells of the necessary nutrients needed to maintain cellular viability, as well as the induction of spectral changes via incubation with a commonly used antineoplastic agent used in the treatment of several cancers, to be described in more detail in Chapter 5. Despite the obstacles presented working in an aqueous environment, spectral data were obtained reflecting changes on the cellular level. Also, these preliminary results were the longest FTIR microspectroscopic data obtained on individual live cells, using a non-synchrotron beam source reported in the literature. Optimization of methodologies could make this
technique useful in the clinical setting as a tool for pre-screening potential drug therapeutics on
cancerous cells; optimization of which is the focus of Chapter 5.

4.5 References:

1. Marcsisin, E. J.; Uttero, C. M.; Miljkovic, M.; Diem, M., Infrared microspectroscopy of

2. Holman, H. Y.; Bjornstad, K. A.; McNamara, M. P.; Martin, M. C.; McKinney, W. R.;
Blakely, E. A., Synchrotron infrared spectromicroscopy as a novel bioanalytical microprobe for

L., Infrared microspectroscopy of biochemical response of living cells in microfabricated

Wood, B. R., Monitoring the reversible B to A-like transition of DNA in eukaryotic cells using
Fourier transform infrared spectroscopy. Nucleic Acids Res 2011

5. Miller, L.; Dumas, P.; Jamin, N.; Teillaud, J.; Miklossy, J.; Forro, L., Combining IR
spectroscopy with fluorescence imaging in a single microscope: Biomedical applications using a

spectroscopic characteristics of cell cycle and cell death probed by synchrotron radiation based


Chapter 5: Optimization of Live Cell Data Acquisition Methodologies

Parts of this Chapter have been submitted for Publication and are included in Appendix II


5.1 Introduction

Discussed in Chapter 3 were the details that distinguished the two data collection modes of the PE 400 FTIR microscope, point mode data acquisition and image mode data acquisition. Both were utilized for experiments presented in this dissertation. This Chapter focuses on how data acquisition and processing methodologies were refined and optimized to allow the faster collection of FTIR microspectroscopic data for live cells. The newly optimized approach provided quality data in shorter collection times with improved S/N compared to the initial point mode collection efforts described in detail in Chapter 4. The optimized approach involves the collection of spectral image maps where every individual pixel in a 1 mm² imaged area represents a spectrum.

Several factors provided the motivation for transitioning the collection of data from point mode aperture based data acquisition to the image mode data acquisition, which included: (1) the point mode data collection technique was manually labor intensive. Every acquired cell required a collection time of over 6 minutes, thereby limiting the number of cells collected for time course experiments. (2) Spectral quality was significantly improved after the transition to acquire
imaged maps. The use of a focal plane array detector increased detection in the low wavenumber region, specifically at the phosphate symmetric vibration 1060 cm\(^{-1}\), near the CaF\(_2\) cutoff. (3) The final advantage in the new approach was the improved spectral image definition and the S/N, made possible with the MATLAB based algorithm, NA-PC reconstruction. All these changes are discussed in the subsequent Sections of this Chapter.

5.2 Image Mode in Conjunction with NA-PC reconstruction

Spectral image maps, containing 20-30 individual cells within 1 mm\(^2\) imaged areas, were collected at 6.25 μm x 6.25 μm pixel size, scanned at 8 interferograms per pixel and 8 cm\(^{-1}\) spectral resolution. A background was acquired for the 16 detector elements before each image was scanned. Each acquired image contained 25,600 pixel spectra. The image mode collection parameters improved data acquisition time in comparison with the point mode methodology. Image collection required a 16-fold fewer number of co-added interferograms per pixel (8 interferograms for image mode vs. 128 for point mode). Also, by collecting at 8 cm\(^{-1}\) spectral resolution rather than 4 cm\(^{-1}\), acquisition time was reduced by a factor 2. The change of spectral resolution between collection methods did not sacrifice the overall spectral quality. Resolution of 8 cm\(^{-1}\) was sufficient and no diagnostic information was lost.\(^1\) In addition, every individual cell collected via image mode consisted of several pixel spectra and the spectrum of the entire cell was obtained by averaging those pixels. Therefore, each spectrum was the average of many spectra, whereas for the point mode methodology each cell was only represented by one spectrum for the entire area. Obtaining several pixel spectra for each cell allowed a more accurate representation of the biochemical makeup of the cell.
A major advantage of collecting data via the optimized approach was the ability to monitor the low wavenumber region, specifically encompassing the symmetric phosphate stretch at 1060 cm$^{-1}$. This was briefly mentioned in Chapter 4, Section 4.3, and was due to the better detectivity, resultant from the smaller area for each of the 16 individual 25 μm x 25 μm detector elements of the focal plane array utilized for image collection. Imaged areas collected at 6.25 μm x 6.25 μm pixel size filled the entire detector element in contrast to the point mode collection, where a 50 x 50 μm aperture was scanned onto a 100 x 100 μm detector area. Each detector element measures 25 μm on edge and is completely occupied even though the pixel is collected from an area of 6.25 μm on edge because the PE 400 infrared microscope employs a 4X image magnification objective. This resulted in better signal from 1800 to 1000 cm$^{-1}$ so the entire spectral fingerprint region could be utilized for data analysis.

Spectral images acquired at the parameters described above allowed for significantly faster collection times. However, this acquisition protocol would not have been feasible without the MATLAB based NA-PC reconstruction routine for data pre-processing, and the PapMap routine for the calculation of cell spectra from the individual pixel spectra. The functions of these algorithms were presented earlier in Chapter 3, Section 3.3.2; however, a more detailed explanation will be provided here. The main purpose of this algorithm was to extract noise information from the imaged pixels representative of only background locations that were devoid of cellular material and to compute a noise profile for the PE 400 instrument. That noise response was then used to extract the noise contribution at every individual wavenumber in each pixel spectrum from the areas containing cellular material.

NA-PC reconstruction was accomplished by computation of two covariance matrices, one for the noise and one for the spectral information of the cells. Signal and noise principal
components were obtained by diagonalization of the respective signal and noise covariance matrices, given by $C_{kl}^S = \sum_{i=1}^{n} S_i^{(\nu)} k S_i^{(\nu)} l$ for the signal covariance matrix, or in matrix notation as $C = S S^T$ where $S$ represents the similarity transform for the spectra, $S^T$ represents the orthogonal principal component transform for the spectra, and $C_{kl}^N = \sum_{i=1}^{n} N_i^{(\nu)} k N_i^{(\nu)} l$ for the noise contributions, where $N$ represents the noise spectra (areas devoid of cellular material).\(^2\)

PCA was performed separately on the cellular data and the noise matrices. Subsequently, the signal covariance matrix was reordered (“noise-adjusted”) using the eigenvectors of the noise matrix\(^3\), such that the spectral information was concentrated in the first PC loading vectors and the noise in the final PC loading vectors. The data were reconstructed using the first 30 PCs, thereby reducing the noise by a factor 3 as previously described.

All noise-adjusted data were imported into the PapMap routine, which reconstructs cellular spectra from individual pixel spectra, subject to the constraints described previously in Chapter 3 Section 3.3.3. Individual cell spectra were converted to second derivatives using a nine point smoothing window function (Savitzky-Golay) and vector normalized. Spectral variance was observed using PCA, all final analysis steps were uniform for both point mode data and image mode data acquired.

### 5.3 Improved Signal to Noise

The new methodology resulted in spectra with improved S/N. The S/N ratio of both point mode and imaged cell spectra was established as follows: the signal was calculated, in absorbance units, as the difference between the baseline at 1772 cm\(^{-1}\) and the maximum of the amide I intensity band at 1652 cm\(^{-1}\) for all individual raw spectra. The noise was calculated using...
the root mean squared noise in the spectral region from 2120 to 2000 cm\(^{-1}\), shown in Equation 5.1 where \(\Delta A_i\) is the absorbance difference between two consecutive positions along the wavenumber axis, \(\bar{A}\) is the mean of all \(A_i\) and \(n\) represents the total number of data points used.  

\[
\text{Equation 5.1} \quad \text{noise} = \frac{\sum |\Delta A_i - \bar{A}|}{n}
\]

Using the absorbance difference \(\Delta A\) between consecutive data points accounted for the upward sloping baseline in all raw spectra. Figure 5.1 illustrates example spectra for different cells collected from (A) an image before NA-PC reconstruction, (B) an image after NA-PC reconstruction and (C) using the point mode data collection methodology for comparison purposes. Table 5.1 shows S/N values of 10 randomly chosen cell spectra before final analysis (at this point, spectra had not been derivatized, smoothed or vector normalized). These data demonstrate that the point mode methodology produced raw data with sufficient S/N (in a more limited wavenumber range, as described above) and that image mode without NA-PC reconstruction produced data with lower S/N. However, noise adjusted imaged data presented a significant improvement.

Another contributing factor to the improved S/N was the difference in the detector utilized, as described in the previous Section. Detector performance is related to area of the detector element. The focal plane array detector employed for image mode data acquisition utilizes 16 individual detector elements, each measuring 25 \(\mu\)m on edge, which is much smaller than the 100 \(\mu\)m x 100 \(\mu\)m area of the point mode single element detector. For that reason the focal plane array detector achieves better detectivity, and therefore better S/N. The true S/N improvement presented by the optimized methodology resulted from the combination of using
Figure 5.1 Signal to Noise Comparison
Shown in this Figure are spectra for individual cells collected via (A) image mode prior to noise adjustment with NA-PC reconstruction, (B) image mode after NA-PC reconstruction and (C) point mode. Good S/N is achieved using the point mode data collection methodology. However, better S/N is achieved using the image mode data collection methodology in conjunction with NA-PC reconstruction. This observation is even more apparent with the values presented in Table 5.1.
Table 5.1 Signal-to-Noise Comparison
This table represents the S/N values computed using the root mean square noise shown in Equation 5.1 and the signal from the intensity value of the amide I frequency band. The S/N was calculated for 10 randomly chosen cells acquired on different dates. The point mode methodology provided acceptable S/N raw spectra; however, collecting the data as spectral image maps and using NA-PC reconstruction to extract noise contributions in the spectral regions significantly improved the S/N.

<table>
<thead>
<tr>
<th>Example cell</th>
<th>Point Mode</th>
<th>Image Mode Pre NA-PC</th>
<th>Image Mode Post NA-PC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell 1</td>
<td>285.5</td>
<td>202.5</td>
<td>645.7</td>
</tr>
<tr>
<td>Cell 2</td>
<td>534.5</td>
<td>307.3</td>
<td>681.8</td>
</tr>
<tr>
<td>Cell 3</td>
<td>603.8</td>
<td>415.2</td>
<td>1027.1</td>
</tr>
<tr>
<td>Cell 4</td>
<td>336.4</td>
<td>394.5</td>
<td>1076.3</td>
</tr>
<tr>
<td>Cell 5</td>
<td>1891.0</td>
<td>334.9</td>
<td>1267.0</td>
</tr>
<tr>
<td>Cell 6</td>
<td>685.8</td>
<td>312.0</td>
<td>946.4</td>
</tr>
<tr>
<td>Cell 7</td>
<td>517.6</td>
<td>417.7</td>
<td>1876.5</td>
</tr>
<tr>
<td>Cell 8</td>
<td>490.4</td>
<td>567.0</td>
<td>1696.3</td>
</tr>
<tr>
<td>Cell 9</td>
<td>577.9</td>
<td>845.0</td>
<td>1671.2</td>
</tr>
<tr>
<td>Cell 10</td>
<td>542.9</td>
<td>954.7</td>
<td>1649.4</td>
</tr>
<tr>
<td>Average S/N</td>
<td>646.6</td>
<td>475.1</td>
<td>1253.8</td>
</tr>
</tbody>
</table>
the focal plane detector in conjunction with the NA-PC reconstruction algorithm and the averaging of the pixel spectral (PapMap) representative of each cell.1

5.4 An Unusual Advantage to Working in Liquid Water: Lack of Dispersive line shapes

Live cell experiments in liquid environments are inherently difficult due to several challenges when working in liquid water. These challenges all deal with path length utilized and include (1) the requirement of short path lengths (2) the inconsistent path lengths between sample and background areas and (3) the difficulties encountered when compensating for path length differences. All these challenges are offset by one significant advantage: the lack of dispersive line shape contributions. Dispersive line shapes are commonly observed in the infrared spectrum of fixed or dried cells and tissue. The line shapes originate from several factors, such as pure reflective components (for transflection measurements), “resonance Mie” scattering and other factors relying on anomalous dispersion of the refractive index.4-6 Observation of dispersive band shapes usually occur at the edge of cells due to addition of a negative intensity at the higher wavenumber side of the absorption spectra, which results in a shift of the absorbance spectra to lower wavenumber values. The exhibited shift can occur up to 25 cm\(^{-1}\) and occasionally reduces the intensity at the amide I band. Previously these dispersive line shapes have shown to be problematic and have complicated the use of FTIR microspectroscopy as a diagnostic approach; however, more recently several correction algorithms have been presented to circumvent the issue.4,6

The enormous advantage to live cell work in aqueous environments is the absence of these dispersive line shapes. The lack of dispersive artifacts is attributed to the similarity in refractive index between a hydrated cell and the liquid environment. This advantage was
recognized during the transition to image mode collection methods, by using a frequency map to illustrate spectral maxima at the amide I and II frequency positions. Illustrated in Figure 5.2 is a (A) frequency map representing one of the collected 1 mm² imaged areas. The blue portion of the map represents areas devoid of cells while the red areas illustrate regions that contained cells. Figure 5.2 (B) shows for the cells imaged that the maximum absorbance in the amide I region coincide at 1652 cm⁻¹. These spectra illustrate the utility of FTIR measurements of cells in aqueous environments due to the lack of dispersive artifacts which are normally present with fixed cells.

5.5 Image Mode to Monitor Drug Induced Spectral Changes

To demonstrate the utility of the refined image mode acquisition methodology, it was applied in similar experiments to those presented in Chapter 4 to detect cyclophosphamide monohydrate induced spectral changes. Before any FTIR results are presented, a brief background on the drug and modes of action will be described.

Cyclophosphamide monohydrate is an antineoplastic agent commonly used in the treatment of several cancers including cervical, ovarian, bladder, brain cancer and several others.⁷⁻¹⁰ The structure of cyclophosphamide monohydrate is shown in Figure 5.3. As with many chemotherapeutic drugs, cyclophosphamide monohydrate presents several risks. A major concern for treatment with this drug is secondary malignancies. Most chemotherapeutic drugs are associated with the risk of development of other cancers.¹¹ For that reason there is a need for a technique, like the one proposed in this dissertation, to pre-screen potential treatments for efficacy prior to administration to a patient’s body to reduce potential risk.
Figure 5.2 Frequency Map Representing Lack of Dispersion for Live Samples
Illustrated in this Figure is the frequency map (A) of the imaged area. The color bar represents the wavenumber at the maxima for each pixel absorbance spectrum. The absorbance spectra for cells shown in A (B), for live cells collected as a 1 mm² imaged area. Unlike fixed or dried samples (C), the maxima at the amide I position of all liquid samples coincided at 1652 cm⁻¹. This negates the need for dispersion correction, which is common practice prior to analysis of dried or fixed samples. The maxima coincide for liquid samples due to the refractive index similarity at the cell/water interface.
Figure 5.3 Cyclophosphamide Monohydrate
This figure demonstrates the structure of cyclophosphamide monohydrate in its pre-activated form. This drug is commonly used in the chemotherapeutic treatment of several types of cancer. It is a highly toxic alkylating agent.
Cyclophosphamide monohydrate is considered an extremely toxic alkylating agent, specifically classified as a nitrogen mustard. This drug is non (cell cycle) phase specific and will target the DNA of a cell independent of its stage in the cell cycle. Cyclophosphamide monohydrate targets DNA by an attack of the 7th position nitrogen atom on guanine bases, shown in Figure 5.4. Most (90%) of the cyclophosphamide monohydrate induced damage occurs as one of three common modes of action: limpet attachments, where one strand is bound to itself in another position isolating part of the DNA strand; cross linking, where two strands become inseparable from one another leading to double strand breakage; or by causing mutations that can lead to nucleotide mispairing and DNA strand fragmentation. Although, most often reported are inter-strand crosslinks.

Figure 5.5 illustrates the processed absorbance and second derivative data for two separately imaged areas. HeLa cells were cultured to CaF₂ windows as previously described in Chapter 3 Section 3.1.1, before subsequent incubation with cyclophosphamide monohydrate. The cells were exposed to the drug by exchanging pure culture medium for culture medium with cyclophosphamide monohydrate (300 μM) and placing them back into the incubator for an additional 12 hour period. The sample window was then transferred to the live cell sample chamber and normal culture medium supplemented with FBS was supplied at a constant flow for the duration of data acquisition. Illustrated in Figure 5.6 is the PCA scores plot demonstrating spectral variance between the cyclophosphamide monohydrate dosed cells in comparison with cells incubated and acquired under normal culture conditions. The variance was observed along PC2, provided in Figure 5.7. From this loading vector two significant conclusions can be made: (1) there was a noticeable spectral change occurring in the low wavenumber region, specifically at the phosphate stretching vibration (1060 cm⁻¹) and (2) there was an anti-correlated spectral
Figure 5.4 Targeted Position on Guanine
Illustrated in this Figure, the 7\textsuperscript{th} position nitrogen, indicated by the yellow star, on guanine is the target for cyclophosphamide monohydrate.
Figure 5.5 Absorbance and Second Derivative Data for Cyclophosphamide Monohydrate Exposed HeLa Cells

Demonstrated in this figure are the absorbance (top) and the second derivative (bottom) spectra for HeLa cells exposed to cyclophosphamide monohydrate. The different colored spectra represent individual cells. These data were obtained using the image mode data collection methodology. These results demonstrate spectral discrimination among the spectral frequencies for the entire spectral fingerprint region, including near the CaF$_2$ transmission cutoff.
Figure 5.6 PCA Scores Plot of Cyclophosphamide Monohydrate Dosed HeLa Cells

The data demonstrated in this figure represent the comparison of HeLa cells exposed to cyclophosphamide monohydrate for 12 hours (red) and HeLa cells kept under normal culture conditions (control, green) for the same experimental duration. There is distinctly detectable variance along PC 2.
Figure 5.7 PC2 for Cyclophosphamide Monohydrate Exposed HeLa Cells
Demonstrated in this Figure is the variance that the cyclophosphamide monohydrate exposed cells exhibit from the cells kept under normal culture conditions, shown in Figure 5.6. The major observations here are the large variance in the phosphate symmetric frequency band region, 1060 cm\(^{-1}\) and the shift occurring in the protein region near the amide I position.
shift occurring in the protein region near the amide I vibration. These observable spectral changes are suggestive for what is known about the mode of action for cyclophosphamide monohydrate. These results demonstrate the potential for using this kind of experimentation and methodology as a tool in the development of personalized cancer treatment plans, specifically to pre-screen potential chemotherapeutic drug treatments on specific cells outside of the body.1

5.6 Summary

This Chapter focused on collection of spectral image maps in conjunction with the newly developed pre-processing algorithm, NA-PC reconstruction, to demonstrate an optimized methodology for live cellular experiments using a non-synchrotron based FTIR approach. Data are now acquired faster allowing larger sampling populations. The ability to acquire more data provides stronger conclusions. NA-PC reconstruction allows noise contributions to be extracted from spectrally diagnostic cellular data by using areas of the image once thought to be wasted information. Every individual pixel is utilized in the noise extraction, including pixels not occupied by cellular content. A significant advantage to performing live cell experiments in liquid culture medium is the lack of dispersive line shapes as a direct result of the similarity in refractive index between a hydrated cell and the liquid environment. Although live cell research is still fairly new in the FTIR microspectroscopic community, provided here were results that present potential for establishing this kind of a technique as a tool in the pre-screening of drug therapies for cancer patients.1
5.7 References


Chapter 6: Conclusions and Future Directions

6.1 Conclusions

The research presented in this dissertation was performed to advance the field of vibrational spectroscopy by utilizing FTIR microspectroscopy as a tool for the detection and evaluation of cellular response to chemotherapeutic agents. The long term intentions of this research are to provide the means for improving standard cancer treatment practices in hopes of alleviating the physical and financial burdens imposed by current practices in drug treatment plan development.\textsuperscript{1, 2} The current protocol for drug treatment typically involves evaluation of drug response after the drug is administered to the patient, and the initially tested drugs are based on response of previous patients with similar conditions and cancer.\textsuperscript{1} As previously outlined, the major flaw in the current approach is that each case usually differs and during the search for the right treatment, damage can be inflicted on the patient’s already compromised health. Sadly, cancer treatments can often be as deadly as the disease.

All results were obtained utilizing a custom built live cell sample chamber which facilitated the collection of spectroscopic data simultaneous to visual image capture.\textsuperscript{3} The live cell sample chamber was maintained at 37 °C and a constant flow of fresh culture medium was supplied to the sample cells. The results of this dissertation illustrated successful detection of induced spectral changes as a result of cellular starvation and as a result of incubation with a commonly used chemotherapeutic agent, cyclophosphamide monohydrate.\textsuperscript{3, 4} Long time course experimentation, as demonstrated in this work with experiments over the course of 24 hours, are essential for a technique that proposes the ability to monitor drug response. Drugs used in the treatment of cancer act on invasive cells \textit{via} several modes of action and on various time scales. The ability to perform long-term experiments provides the ability to truly evaluate response to
the fullest extent, from induction to cellular death. The methodologies used in this research transitioned from point mode data collection to image mode data collection in conjunction with MATLAB based analysis algorithms to generate faster live spectral data with improved spectral quality.

FTIR microspectroscopy of live cells is an emerging focus in the FTIR spectroscopic community. As a result, there are several aspects of live cellular studies still to be addressed before this type of technique can be integrated into clinical practices for the development of personalized cancer treatment plans. Sample preparation and data collection methods are not common. Sample chambers are developed independently by research groups to accommodate available instrumentation. Also, several groups are utilizing synchrotron sources. As discussed in Chapter 2, synchrotron sources are not practical in cost or size with respect to the laboratory or the clinical setting. For this reason, all experimentation in this dissertation was conducted using a commercially available source.

6.2 Future Directions

Future experiments to expand the realm of this technique include evaluation of several different chemotherapeutic drugs in live cells. This type of proposal could provide useful connections between mechanisms of different available compounds to establish which drugs act similarly on cancers. Knowing which drugs force cells to respond similarly would present a means for developing substitutions for drugs to eliminate those that present higher occurrences of unfavorable risks (secondary malignancies, leukemia, etc.).

Another potential area for future FTIR experimentation on live cells is the evaluation of combination chemotherapy. Combination chemotherapy is used to lower the toxic effects of
many chemotherapeutic drugs. Live cell experiments could evaluate combinations of treatments to determine lowest drug dose(s) necessary to achieve a response. This would assist in eliminating unnecessarily high drug doses and potentially decrease risk involved in treatment plans.

Future goals also include further optimization of technique and methodologies. Optimization includes improvements of sample chamber design and collection methodologies. An ideal design would be one that could allow viability testing via assays or staining methods. For example, Trypan blue is a stain for viability that indicates cell death; however it can not be applied with the current cell chamber design. The reason is that the top window cannot be removed without damaging the cells and also the stain can not be introduced to the assembled sample chamber because there is no way to completely flush out all traces of culture medium. Culture medium trapped at the sample (even miniscule amounts) can produce a false result. Also, as science progresses and equipment and instrumentation improve, it is important to re-evaluate experimental techniques to utilize available resources to their fullest extent. An improvement to the instrumentation utilized for this research would be replacement of the source with a hotter source to obtain higher S/N ratios. The interrogation of live cells could eventually lead to more desirable modes of treatment of patients in their diagnoses and treatments for disease.

6.3 References:


APPENDIX I

Publication

Special focus issue: Optical Diagnosis
Infrared microspectroscopy of live cells in aqueous media†

Ellen J. (Swain) Marcsisin,* Christina M. Uttero, Miloš Miljković and Max Diem

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Fourier Transform Infrared (FTIR) spectroscopic measurements of individual, live HeLa cells in culture and buffer media are presented. Spectral data were acquired using a newly designed live cell chamber developed in the authors’ laboratory. Data were processed using MATLAB-based routines that correct for the overcompensation of water encountered during live cell measurements in aqueous samples. Data presented are from live cells monitored over an extended period of time as well as a comparison of live cells exposed to perturbing conditions.

Introduction

FTIR microspectroscopy has become an increasingly valuable method for interrogating the biochemical signatures associated with the onset of disease in eukaryotic cells. This method provides the sensitivity necessary to probe biological samples on the single cell level. Even though this technique has been broadly applied with respect to biologically static (i.e., fixed) diagnostics, there is still plenty of room for advancement to be made applying this methodology to the area of live cellular samples.

When a patient is diagnosed with disease, there are crucial windows of time for effective treatment; therefore, a valuable implication of live cell microspectroscopy would be the opportunity to quickly and efficiently identify the cause of disease and screen a broad arsenal of possible therapeutic agents. Applying microspectroscopy to live cells exposed to drugs offers the ability to observe spectral changes associated with specific drugs and assign those changes to specific biochemical cellular events.

In the case of cancer, treatment optimization could be achieved by excising tumor cells, propagating them in culture, and testing chemotherapeutic possibilities on the cells specific to a patient. FTIR microspectroscopy would allow doctors and clinicians to provide patients with customizable or designer treatment plans, which would decrease the cost of unnecessary procedures and therapies as well as improving patient prognosis. This technique would help with finding effective treatment options before unsuccessful procedures are utilized that can be painful, invasive, mentally oppressive, and have serious side effects.

Previously, two groups reported on their efforts to acquire spectroscopic data for cellular samples in buffered aqueous medium, thus demonstrating the possibilities for live cellular studies. Their research established the potential to acquire useful IR spectroscopic data in aqueous environmental conditions even though the strong water deformation band at 1620 cm⁻¹ distorts the normal band profile of a cell in the amide I and II regions. Miljković et al. described spectra where the water contribution was subtracted using a background spectrum collected nearby the cell of interest, resulting in a more normal looking cellular spectral profile. This is similar to work done much earlier using the strong water association band at 1625 cm⁻¹ to subtract water contributions from IR spectra of proteins. Prior to that research, the principle of using water bands as internal standards for spectral subtraction was introduced by research performed on proteins in solution. Several others have reported their efforts to collect spectroscopic measurements on various live cell cultures where they observed apoptosis, necrosis and cellular damage using a variety of FTIR modes and sample chambers on live and fixed cells.

Various studies have reported spectral changes of fixed cells as they undergo internal biochemical changes, both naturally and/or due to applied stresses. These studies have demonstrated that FTIR microspectroscopy provides the sensitivity necessary to differentiate between the different stages of a cell’s division cycle. Similar work has been performed on live cells during specific cellular stages during the cell cycle. More recently, other research groups have reported the signature spectral features specific to individual drugs and their mechanisms of action using fixed samples. Useful biochemical information could be extracted in real time by evaluating cellular signatures throughout progression of cell cycles, and as cells are exposed to chemically altering compounds.

The results presented in this manuscript are FTIR spectroscopic data collected continuously for hours on individual live HeLa cells, and the methodology used to suppress confounding factors such as the strong absorption of water in the IR region and the compensation from the CaF₂ windows in background spectra. We have demonstrated that the continuous observation of individual, live cells over extended periods of time is possible and practical, both under conditions that promote cellular proliferation and conditions that result in cellular death, i.e. apoptosis and necrosis. The cellular response as a result of medium starvation will be presented.

† This article is part of a themed issue on Optical Diagnosis. This issue includes work presented at SPEC 2010 Shedding Light on Disease: Optical Diagnosis for the New Millennium, which was held in Manchester, U.K. June 26th–July 1st 2010.

* Infrared microspectroscopy of live cells in aqueous media.
Experimental

Sample preparation

HeLa cells (ATCC, Manassas, VA) were cultured in 75 cm² culture flasks (Corning, Lowell, MA) using minimum essential Eagle’s medium (ATCC, Manassas, VA) supplemented with 10%, by volume, fetal bovine serum (FBS) (ATCC). Cultured flasks were incubated at a constant 37 °C and kept in an atmosphere of 5% CO₂. Cells were cultured until confluent, and removed from the flasks using trypsin-EDTA (ATCC). Cells were then re-seeded onto special CaF₂ windows (see below) in 47 mm culture dishes (Millipore, Billerica, MA) with fresh culture medium supplemented with 10% FBS and placed back into incubation for approximately 12 h.

Transmission measurements of live cells were carried out using a live cell chamber designed in the authors’ laboratory. The live cell chamber, Fig. 1, consists of three main components: (1) a Plexiglass stage plate that is interchangeable with the Perkin-Elmer Spotlight 400 series (PE 400) stage plate, (2) an aluminium top plate housing a 32 mm sample cell consisting of a 32 mm × 3 mm window, drilled with two 1.5 mm holes 18 mm apart for culture medium flow, and a second CaF₂ window (Biotools, Inc., Wauconda, IL) that has an approximately 10 μm deep, etched well providing a space large enough for the cells to remain intact, Fig. 2. The culture medium is delivered to the cells via an IV-style gravity driven drip which is adjustable with respect to the rate of flow. For experimental purposes, the flow was kept below 4 μL/s. The temperature at the windows was closely monitored and maintained at 37 °C.

Fig. 1 Schematic of the live cell chamber designed in the authors’ laboratory, not drawn to scale. Top: cross-sectional view of chamber and Bottom: top view of the chamber, where culture medium flow (A) is constant and regulated using an IV-like regulator (B) to the cells via an inlet (C) and disposed via an outlet (D) in the bottom plate (G). The live cell chamber is constructed from aluminium and the temperature at each window is maintained at a constant 37 °C by two heating cartridges (E) housed within the top plate (F). The cells in the culture medium are contained in a sample compartment, shown in light blue, and detailed in Fig. 2.

Data acquisition

FTIR transmission data (in single beam mode, see later) were acquired using a Perkin-Elmer (Shelton, CT) Spectrum One/Spotlight 400 infrared micro-spectrometer in point mode using a 100 μm single element HgCdTe detector. The instrument bench top and microscope stage area were constantly purged with −40 °C dew point dry air to reduce atmospheric water vapor spectral features.

Background spectra were first collected in an area adjacent, but unobstructed by the cells on the substrate. Subsequently, a cell on the substrate was visually selected, and its spectrum was acquired, using the same instrument background. This spectrum is referred to as the ‘raw cell spectrum’. The cells’ absorption spectra were calculated as discussed below (see ‘Data correction and processing’ section below). For the collection of raw cell and background spectra, 128 interferograms at 4 cm⁻¹ resolution were collected using a 50 μm × 50 μm aperture, about the same area of a HeLa cell.

The first experiment was a study of 13 individual HeLa cells, scanned at 5 time points. The first data collection time point was 30 min after transfer of the sample window to the live cell chamber and the last collection time point was 10 h and 45 min post-transfer. The chamber and second CaF₂ window were pre-warmed to 37 °C. Cells received a constant flow of fresh culture medium throughout the duration of the experiment. Time points were approximately 2–2.5 h apart and a water spectrum was collected adjacent to each cell before data were collected.

A cellular starvation experiment was performed where cultured cells were deprived of growth medium. The chamber and top window were pre-warmed to 37 °C before transfer of the cells from the incubator. When cells were placed into the chamber, the culture medium was exchanged with phosphate buffer solution (PBS). The first collection time point was 30 min after transfer of the window to the chamber, followed by 4 other time points that were spaced approximately 2 h apart. This experiment was planned to mirror the data collection time points of the first experiment.

A third experiment, conducted over the course of 24 h with the same conditions as the first experiment, was performed to demonstrate that (a) results from the first experiment, which followed 13 cells for 11 h were legitimate with respect to the amount of time the cells were kept in the live cell chamber and (b) cells were exhibiting signs of cellular death due to deprivation of light.
culture medium and not as a result of conditions imposed by the live cell chamber itself. This third experiment was a study of 20 individual HeLa cells in normal growth medium, scanned at 10 time points over the course of 24 h (beginning with removal of the sample window from the incubator and finishing with the collection of data for the last cell in the final time point).

Data correction and processing

A data processing routine, using a graphical user interface (GUI), see Fig. 3, was developed in the authors’ laboratory using the MATLAB (The Mathworks, Natick, MA) environment to correct for water overcompensation by calculating a scaled ratio of the ‘raw cell spectrum’ and the ‘background spectrum’. This step is necessary since the concentration of water is higher when measured at a location outside a cell, as compared to within a cell. This overcompensation creates an abnormal band profile in the protein region of the spectrum, making the amide I to amide II peak intensities look approximately equal. The water correction routine uses the water combination band at 2125 cm\(^{-1}\) as an internal standard for water subtraction.\(^{4}\) A scaling factor, \(SF1\), is applied to the background water spectrum, \(bkgd\), for the calculation of the absorbance, eqn (1a) and (1b). This factor is adjusted graphically until the broad peak at 2125 cm\(^{-1}\) is eliminated, i.e. until the area underneath the peak is minimized. However, the water background spectrum is also affected by the transmission cutoff of the two CaF\(_2\) windows, which causes a spectral distortion below 1100 cm\(^{-1}\). This can be compensated for by introducing a third component to the original equation, the single beam spectrum of CaF\(_2\), and applying an opposite scaling factor, \(SF2\), eqn (1c). The corrected spectra are then smoothed using the Savitsky–Golay smoothing algorithm (13 point window) and vector normalized in the spectral region of 1000–3000 cm\(^{-1}\).

\[
CellAbs = -\log\left(\frac{\text{raw cell}}{bkgd}\right) \quad (1a)
\]

\[
CellAbs = -\log(\text{raw cell}) + (SF1 \times \log(bkgd)) \quad (1b)
\]

\[
CellAbs = -\log(\text{raw cell}) + (SF1 \times \log(bkgd)) + (SF2 \times \log(\text{CaF}_2)) \quad (1c)
\]

Results and discussion

The results and experiments being presented reflect the implementation of our live sample chamber to collected FTIR spectroscopic data on individual living HeLa cells in an easily manipulated cellular environment. By using this chamber, experimental conditions, such as temperature and nutrient supplementation to the cells, can be manipulated. In the following experiments several cells were continuously monitored over recorded time points allowing the observation of spectral changes a result of time and as a result of altered experimental conditions.

The collection of spectroscopic measurements on live cells using the mid-IR range is a daunting task due to the strong absorbance of water in the amide I and amide II spectral region of cells, which reduces the overall transmission of the sample, and tends to distort the amide I/II band intensity ratio. By using the GUI-based correction routine developed in the authors’ laboratory (see the Experimental section for details), cellular data acquired in aqueous medium can be ‘background’ corrected with respect to the water absorption. This correction is necessary because the amount of water volume present in the background is greater than that of the cellular position.

Since these cells are live, their spectral features, and those of the adjacent areas, are subject to constant biochemical changes. Therefore, it is necessary to collect background spectra prior to scanning each cell at every time point. If a common background file was used for every ratio, the results would not accurately reflect the sample in that moment because one would not have the direct representation of the environment around a cell at that given moment. Thus, each cell spectrum was obtained using a ‘background spectrum’ collected at the same time point in the vicinity of the location from which the ‘raw cell spectrum’ was collected.

Fig. 4 represents the operation of the data correction methodology, and indicates the quality of data that can be collected using a standard infrared micro-spectrometer for cells in aqueous media. Fig. 4(A) shows the spectrum, referred to as \(CellAbs\) in eqn (1a), of a live cell in normal growth medium (blue trace). After the water and CaF\(_2\) background compensation (i.e., \(CellAbs\) in eqn (1c)), the spectrum shown as the red trace was obtained. The black arrow indicates the water combination band that was used as an internal standard for the correction, and the spectrum was adjusted until the area underneath the peak was approximately equal to zero. In order to assess the effect of the water compensation on the amide I and II bands, we show in Fig. 4(B) the second derivative spectra for both spectra in Fig. 4(A), demonstrating that the frequencies of the cell spectrum
remain unchanged, thus maintaining the spectral integrity of the data. However, an intensity difference is observed in the second derivative spectrum upon water background compensation, indicated by the black arrow just above 1600 cm\(^{-1}\), also indicated by a black arrow.

One of the most exciting possibilities provided by live cell studies is the ability to monitor in real time the biochemical changes taking place in cells as a result of an administered compound or drug. In order to conduct such an experiment it is necessary to sustain cellular life for prolonged periods of time. Presented in Fig. 5 are the results collected during an observation of 13 individual, live HeLa cells over the course of 11 h. The experimental time (\(t = 0\)) was set as the cells were removed from the incubator and placed inside the live cell chamber, and the last time point (\(t = 525\) min) was the time that the single beam spectrum was collected for the first cell of the 13 cells in the final time point. Fig. 5(B) represents the second derivative spectra from each time point, averaged over all 13 cells. Fig. 5(A) is a principal component analysis (PCA)\(^{14}\) scores plot of the data collected from the same 13 cells at 5 different time points. The data used to generate the PCA scores plot were uncorrected with respect to the water contribution; however, the data were smoothed using the Savitsky–Golay algorithm (13 points) and vector normalized. The time points, shown in the legends, represent the time that the first cell of 13 cells was scanned, i.e. cell 1 data were collected 30 min after removal from the incubator followed by cells 2 through 13. Subsequently, cell 1 data were recollected 150 min after removal from the incubator followed by the recollection of cells 2 through 13 and so on for the remaining 3 time points. The lack of clustering of the 65 cell/time points in the PCA scores plot suggests that the 13 cell remained unchanged and alive during the course of the experiment.

Eukaryotic cells are sensitive entities that biochemically reflect perturbations to their environments. Vibrational spectroscopy can be used to detect those biochemical changes. One example of disrupting a cell’s environment would be depriving it of the nutrients necessary for sustaining life. In a separate experiment, the constant flow of fresh culture medium and nutrients was replaced with phosphate buffer solution (PBS) and the experiment was performed with time points consistent with the long term live cell study mentioned above. Fig. 6 shows a gradual trend between data collected for cells approximately 30 min after their removal from normal growth medium and then measured over the course of 4 more time points, spaced 2–2.5 h apart. The data were uncorrected with respect to the water contribution, but were smoothed using the Savitsky–Golay algorithm and vector normalized. Time points in the legend are as previously described. Data were analyzed, as described above, via PCA. The last PCA scores plot, shown in Fig. 6(A), indicates that some cells...
in the two last time point spectra (390 and 525 min) start to differ along PC2 from the earlier time points. The 270 min time point, shown by red squares, appears to be the time after which spectral changes are discernible.

Cell morphology also was followed visually to correlate spectral and morphological changes. It is apparent that the individual cells began to shrink, as would be expected with cellular death. Fig. 7(A–E) depicts these morphological changes at the corresponding five time points. For the first three time points (A–C) there are no significant visual changes: the outer cell membrane appears intact and well defined. In Fig. 7(D and E) the outer membrane is less discernable and it becomes apparent that the overall size and shape of the cell has decreased and become more round. These morphological observations are consistent with common knowledge of cellular death, and agree with the PCA-based results that indicated that the last two time points exhibited different spectral characteristics.

The final experiment, performed at the suggestion of a referee, following the cells over the course of 24 h in normal growth medium, provides additional evidence that the cells are in fact viable during experimentation. Fig. 8 demonstrates the PCA scores plot of 20 individual HeLa cells at 10 time points. No clustering was observed indicating that spectrally no significant cellular changes are taking place. Unlike the cells involved in the culture medium deprivation experiment where cell culture medium was replaced with PBS, the cells involved in the 24 h experiment show no drastic visible morphological changes. This also suggests that the cells involved in this study are not undergoing cellular death. In these 20 cells we did not observe any cells dividing, it may be that these cells have entered a quiescent state and are not actively dividing within the chamber at the time of the experiment. These data represent one of the longest transmission experiments ever performed on individual live cells using FTIR microspectroscopy to the knowledge of the authors.

These results are preliminary and further experiments are underway.
Conclusion

In this study, we report preliminary work using FTIR microspectroscopy on individual, live HeLa cells using a live sample chamber, designed in the authors’ laboratory, which enables spectroscopic measurements in aqueous medium. We also described our approach to overcome the strong absorption band of water that causes an abnormal band profile in the protein spectral region of cells. This methodology, using the broad water peak at 2125 cm$^{-1}$, shows that data collected are representative of individual cells in aqueous medium and despite the strong water absorption band, comparative analysis can still be conducted on the second derivative of the data that is not water corrected because the spectral frequency locations remain unaffected. Finally, we demonstrate that cell death, induced by starvation, induces spectral changes that are correlated to observable morphological changes.

Acknowledgements

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References

2 A. Derenne, R. Gasper, A. Benard and E. Goormaghtigh, Effect of different anticancer drugs on prostate cancer PC-3 cells classified by FTIR imaging Poster session presented at: SPEC 2010 Shedding Light on Disease: Optical Diagnosis for the New Millennium, 2010 June 26-July 1, Manchester, UK.
3 M. Miljković, M. Romeo, C. Matthaeus and M. Diem, Infrared microspectroscopy of individual human cervical cancer (HeLa) cells suspended in growth medium, Biopolymers, 2004, 74(1–2), 172–175.
APPENDIX II

Publication

Noise Adjusted Principal Component Reconstruction to Optimize Infrared Microspectroscopy of Individual Live Cells

by

Ellen J. (Swain) Marcsisin, Christina M. Uttero, Antonella I. Mazur, Miloš Miljković, Benjamin Bird and Max Diem

Department of Chemistry and Chemical Biology, Northeastern University, Boston, MA 02115

Summary

We have optimized an imaging methodology capable of monitoring individual live HeLa cells using non-synchrotron FTIR in an aqueous environment. This methodology, in combination with MATLAB based pre-processing techniques, allows fast and efficient collection of data with high signal-to-noise in comparison with previous methods using point mode data collection, which required manual operation and more collection time. Also, presented are data that illustrate interpretable spectral differences from live cells treated with chemotherapeutic drugs, demonstrating the potential of this methodology to develop more desirable modes of treatment for patients in their diagnoses and treatments for disease.

Introduction

Infrared microspectroscopy (IR-MS) has demonstrated itself to be a useful technique in the area of disease interrogation and diagnosis.1-5 In the last decade, attention has been devoted towards harnessing this technique for applications investigating live cellular samples6-11 which has proven to be extremely challenging. In order to perform spectroscopic measurements on live samples, it is necessary to conduct experiments in an aqueous environment which leads to significant obstacles in the collection and interpretation of data due to strong water absorption bands in spectrally valuable diagnostic regions.7, 8, 11-14 Using IR-MS to interrogate disease (i.e. cancer) on live cells provides the potential for revolutionizing procedures for patient treatment by developing ‘personalized cancer treatment’ plans that would allow a physician/clinician to harvest viable cells from a patient’s diseased organ, propagate the cells in culture and use a flow through device that sustains cellular life while monitoring the effect of chemotherapeutic agents spectro-
This would allow evaluation of different potential treatments available to a patient before introducing agents with harmful effects directly to the body ultimately saving time, money and the health of the patient. Similar studies have recently been performed involving the investigation of anticancer drugs on PC-3 prostate cancer cells by observing FTIR spectral changes in cells, suspended in isotonic NaCl solution, which were treated with structurally related chemotherapeutic compounds.

In order for IR-MS to be useful for collecting data on live cells, it is necessary to achieve fast data acquisition with high signal-to-noise (S/N) to detect small changes in the cellular spectra brought on by drug treatment. Data acquisition time is critical when collecting live cells because data on more cells can be acquired at different time points of the experiment, resulting in better sampling statistics. Previously, some authors have claimed that such measurements are practical only with synchrotron sources.

Here we present successful efforts of data collection using non-synchrotron FTIR in combination with pre-processing techniques that make collection of individual live HeLa cells fast and efficient with good S/N. We also demonstrate a progression and comparison of the various methods and processing techniques that our laboratory has used to emphasize the reason we believe our latest efforts result in optimal live IR-MS data collection. Finally, we present data that illustrate interpretable spectral differences from live cells treated with chemotherapeutic drugs.

**Experimental**

**Sample Preparation**

HeLa cells (ATCC, Manassas, VA) were cultured in 75 cm$^3$ culture flasks (Fisher Scientific, Pittsburgh, PA) for a minimum of 24 hours post-thaw from storage at -80 °C. Cells were cultured using minimal essential Eagle’s medium (ATCC) supplemented with 10% fetal bovine serum (FBS) (ATCC). Culture medium was incubated at 37 °C, 5% CO$_2$. As the flask reached confluence, cells were removed from the flask using trypsin-EDTA solution (ATCC) and re-seeded onto a CaF$_2$ window (International Crystal Laboratories, Garfield, NJ) designed specifically to fit into a flow through cell chamber designed in-house which accommodates live cell spectroscopic measurements (Figure 1). Cells were cultured on the windows for approximately 12 to 24 hours to assure cells were not clustered together (less than a complete cell cycle, 24
The flow-through cell chamber design fits into a custom designed stage plate, made of Plexiglass, to minimize heat transfer to the microscope stage of the spectrometer. The CaF$_2$ window with the attached cells was covered by a second CaF$_2$ window with an inset well (Biotools, Inc, Wauconda, IL), path length approximately 6-10 μm (Figure 1). The two CaF$_2$ windows were placed between two aluminum plates, one of which accommodates the flow of culture medium to the cells on the window, and the second houses two heating cartridges maintained at 37 °C by an external power supply. Fresh culture medium supplemented with FBS was delivered to the cells by an IV-like setup with a controllable thumb regulator.\textsuperscript{11}

The preliminary drug exposure experiments were conducted with cyclophosphamide monohydrate, an alkylating agent commonly used in chemotherapeutic therapies for various cancers.\textsuperscript{16-18} HeLa cells, seeded onto a CaF$_2$ window as described above, were exposed to high cyclophosphamide concentrations (394 μM) for 12 hours in culture medium before transferring the window to the live cell flow-through cell chamber for data collection.

\textit{Data Acquisition}

FTIR measurements were performed using a Perkin Elmer (PE) Spectrum One/Spotlight 400 (PE 400, Shelton, CT) instrument. The PE 400 is capable of performing measurements in single point mode, using a 100 μm single element HgCdTe detector. For all point mode data acquired, a 50 μm x 50 μm sized aperture was used on all cells with 128 background scans and 128 single beam scans for each cell at 4 cm\textsuperscript{-1} resolution. A ratio of each cell against the background was performed using an algorithm developed in MATLAB (The Mathworks, Natick, MA) to obtain an absorbance spectrum.\textsuperscript{11} The background was collected adjacent to each cell, in a location that was free of cellular material.

The PE 400 provides image mode data acquisition using a 8 x 2 detector element focal plane HgCdTe array detector, each detector element measures 25 μm on edge. The detector elements are operated in photoconductive mode, with a duty cycle of nearly 100%. The sample image can be magnified by a factor of 4 such that each detector element samples an area of 6.25 μm x 6.25 μm. All image mode data were collected as 1 mm x 1 mm images using a 6.25 μm x 6.25 μm pixel size, with 120 background scans and 8 co-added interferograms per pixel at 8 cm\textsuperscript{-1} resolution. The spectral region collected for both methods was 1000-3000 cm\textsuperscript{-1}. The low wavenumber cutoff of 1000 cm\textsuperscript{-1} was dictated by the CaF$_2$ transmission, rather than the detector
sensitivity. The instrument bench and stage area were constantly purged with -40 °C dew point dry air to reduce atmospheric water vapor contributions.

Data presented here were acquired within a few hours to assure viability of the sample, although we have previously demonstrated that this flow through chamber is capable of sustaining live cells for at least 24 hours.\textsuperscript{11} Data acquired in point mode required approximately 6 minutes per cell, whereas data acquisition for a 1 mm x 1 mm image lasted approximately 34 minutes per image, which typically contained between 20 and 30 cells. A comparison of these methods will be further discussed to demonstrate the improvements made in our experimental methods.

Data Processing

All data pre-processing and analysis were done using algorithms developed in MATLAB (Mathworks, Natick MA) software. Two entirely different procedures for data processing were developed for point-mode and imaging mode data acquisition.

Point Mode Pre-Processing

In point mode data collection, 128 co-added interferograms for each cell and 128 co-added interferograms for each background location were ratioed, without liquid water correction, to obtain the absorbance spectrum of each cell. We have discussed the compensation methods to correct for liquid water spectral contamination in detail in a previous publication.\textsuperscript{11} The water correction imparts a more ‘normal’ looking band profile to the spectra, compared to cells that are from dried or fixed samples;\textsuperscript{11} however, this correction is largely cosmetic in nature and does not shift the amide I peak frequency of the second derivative spectra, although it does affect the amide I band intensity somewhat. If liquid water compensation is utilized, the water combination frequency band, at 2100 cm\textsuperscript{-1}, is used as an ‘internal standard’ to minimize water spectral contributions.\textsuperscript{11,14} This band does not overlap cellular spectral features, so it can be used to scale the water spectrum such that the water deformation band at \textit{ca.} 1620 cm\textsuperscript{-1} can be eliminated.

If all spectra are processed equally, water compensation does not affect or improve the ensuing analysis by chemometric methods, specifically Principal Components Analysis (PCA).\textsuperscript{19} More important than the liquid water compensation are minor fluctuations in the atmospheric water vapor contributions, which lead to variations in the rot-vibrational water vapor back-
ground. Even when not visible to the naked eye, variations in the water vapor background may be detectable in the PCA loading vectors which may influence the clustering and does not represent biochemical changes in the sample.

**Image Mode Pre-Processing**

All 1 mm x 1 mm imaging data sets consist of 25,600 spectra and are pre-processed by a method known as Noise Adjusted Principal Component reconstruction (NA-PC). The implementation of NA-PC used in the authors’ laboratory was written in MATLAB (Mathworks, Natick, MA). Images in PE 400 format (.fsm files) are loaded, baseline offset and integrated in the amide I region, 1626-1676 cm\(^{-1}\), to yield a raw intensity map of the raw data (see Figure 2B). This map is used to discriminate areas of the image that contain cells from those that only have water contributions. These latter areas are used to construct a “noise correlation matrix”, whose eigenvectors matrix is stored for the next step.

Next, a matrix is calculated that is based on pixel areas that contain cellular materials only, again using the amide I intensity criterion. Images are shown in Figure 2 that depict both areas of the water background (A) and of cellular material (B). PCA is performed on all spectra of cellular material, and the resulting eigenvector matrix is resorted using the eigenvector matrix of the noise correlation matrix.

Noise adjusted spectra are reconstructed from the resorted first 30-50 loading vectors. For our analysis, 30 principal components (PC’s) were chosen which reduced the noise level in the spectra by a factor of 3 (roughly equivalent to a 9 point Savitzky-Golay moving window smoothing function). One of the major advantages of NA-PC over standard smoothing functions is that the noise is reduced over the entire spectral range based on the amount of noise at each wavenumber. For the PE 400 instruments, noise is much larger in the high wavenumber region (4000 cm\(^{-1}\)) than at 1000 cm\(^{-1}\), mostly due to detector response and source intensity distribution. NA-PC constructs a noise matrix that accommodates the instrument noise profile, whereas a smoothing filter function will reduce the noise level independently of the S/N at each wavenumber. Thus, the smoothing filter will over compensate the low frequency region in order to provide good noise reduction in the high frequency region.

Following NA-PC, data is processed by the PapMap algorithm, which co-adds pixels belonging to each individual cell. This is accomplished by constructing a binary mask of the en-
tire image in which contiguous areas belonging to one cell are constructed based on the amide I intensity of the data matrix. All pixels identified by the binary mask to belong to one cell are co-added to produce the final cell spectrum. The user can select the number of pixels considered to be the minimum or maximum of spectra belonging to one cell. Areas and coordinates of the spectra deemed to belong to one cell are calculated and coordinates for each cell are stored for later correlation purposes. The final outcome is a database representing average cell spectra with their cell numbers and corresponding cell coordinates relative to one another.22

The remaining analysis process is uniform for both point mode data and image mode data. All spectra are converted to second derivative using a Savitsky-Golay 9 point derivative function and vector normalized prior to further analysis via PCA.

Here we focus primarily on the pre-processing techniques used to demonstrate our methodology for obtaining IR-MS data on live cells. In order to demonstrate that this methodology is comparable to earlier results, we have performed signal-to-noise (S/N) calculations prior to the final processing steps on data from the point mode collection, raw images before NA-PC, and after NA-PC. The signal was calculated using the difference between the maximum of the amide I band at 1652 cm\(^{-1}\) and the baseline at 1772 cm\(^{-1}\), and the noise was calculated as the noise in the wavenumber region between 2000 and 2120 cm\(^{-1}\), Equation 1. \(A_i\) represents the absorbance difference between each consecutive position along the wavenumber axis, \(\bar{A}\) is the mean of all \(A_i\) and \(n\) represents the total number of points used. The reason for using the absorbance difference between each consecutive position as the \(A_i\) value was to compensate for the upward slope of the baseline.

**Results and Discussion**

Previous data collected using the live cell chamber were acquired using point mode and have relatively good S/N, providing motivation for further experimentation on individual live cells. The main drawback with the original methodology was the duration of a single experiment with respect to the amount of data being collected since data collection for each cell required approximately 6 minutes. Thus, it took about 2 hours to collect spectra for ca. 20 cells; we feel that this number represents the minimum number of cellular spectra to be collected in time dependent studies. However, significant changes in cell biology can occur within 2 hours; they could easily transition from one stage of the cell cycle into another.
This methodology has since been replaced, and all data acquired are collected using image mode. One individual 1 mm x 1 mm image map usually covers 20-30 individual cells (in some instances more than 30 cells) and can be collected in approximately 30 minutes, independent of the number of cells contained in this area. The major advantage of this methodology is that the time being spent collecting data from areas not occupied by cells can be used to improve the S/N of the cell spectra via NA-PC. Presented in Figure 3 are three individual cellular spectra, two of which are from the same cell, collected via image mode with and without NA-PC reconstruction, and the third represents an individual cell collected via point mode using a 50 μm x 50 μm aperture. NA-PC processing produces data with S/N comparable or better than that of data collected via point mode using a 16-fold fewer number (128 v. 8) of co-added interferograms per pixel. We attribute the increase in S/N ratio mostly to the 16-fold smaller area of the individual elements of the array detector elements, and due to the fact that the array data were collected at 8 cm⁻¹, rather than 4 cm⁻¹ resolution.

Table 1 illustrates the S/N improvement for 10 cells’ spectra, chosen at random, and at the bottom the corresponding average S/N calculation for each method. The S/N calculations for the image mode spectra before and after NA-PC reconstruction are from the same cell and the point mode data are of a different cell (point mode collection and image mode collection were not performed on the same sample, but on samples prepared in the same manner). Although point mode acquisition results in data with acceptable S/N; however, it is faster to collect data using image mode followed by NA-PC reconstruction.

Figure 4 represents two separate locations scanned as six separate imaged areas. The areas are shown in relation to one another where imaged area’s 1, 2 and 3 were scanned consecutively in the same location on the stage, followed by imaged areas 4 and 5 at a separate location and then finally imaged area 6 at the original stage location (illustrated in Figure 4D). Figure 4A and B show the raw and processed data used for analysis consecutively.

The scores plot seen in Figure 4C shows that the six imaged areas collected successively on the same sample window cluster on top of one another showing no spectral variance. This is due to the conscious effort to avoid PC’s representing the strong rot-vibrational contributions of atmospheric water vapor. PCA is sensitive enough to detect the differences in the rot-vibrational contributions specific to atmospheric water vapor at the time one imaged area is scanned in comparison with the next imaged area. If an eigenvector representing those rot-vibrational differ-
ences is used in the presentation of a scores plot, variance unrelated to biochemical contributions would be observed creating a false result. Alternatively, by not reviewing the PC’s prior to demonstrating the data via a scores plot, using a PC indicative of water vapor, the separate images would no longer cluster together, as would be expected of cells of the same sample, but the spectra would cluster based on their individual image files as a result of changes in the atmosphere during the collection of each image (scores plot not included).

Next, we turn to the discussion of a peculiar advantage of collecting cellular spectra in an aqueous environment. Often, only the disadvantages arising from water interferences are discussed which include the need for short path lengths, the problem of maintaining constant path lengths between sample and background measurement, and the difficulties of compensating for the water contribution between sample and background spots. However, all these difficulties are accompanied by an enormous advantage of working in an aqueous environment: the absence of dispersive band shape contributions which often are observed in the infrared spectra of cells and tissues. The origin of these reflective band shapes is now well understood and includes pure reflective components (particularly in transflection measurements), “resonance Mie” scattering, and other effects that depend strongly on reflective index, which undergoes anomalous dispersion within regions of absorption. The reflective band shapes are most commonly observed at the edges of cells and tissues; due to the addition of a “negative” intensity at the high wavenumber side of the absorption spectra, the absorption maxima are shifted toward a lower wavenumber (by up to 25 cm\(^{-1}\)), and the amide I peak is significantly reduced in intensity. These dispersive line shapes have presented enormous problems in the past, and until recently, have seriously reduced the utility of IR-MS as a diagnostic tool.

A significant advantage to live cell work is the absence of these effects. This can be attributed to the fact that the difference in refractive index between an individual hydrated cell and water is so small that scattering effects can not be detected. This is demonstrated in Figure 5 which shows a 1 mm x 1 mm image map of individual live cells depicting the frequency of the amide I position which occurs consistently at 1652 cm\(^{-1}\), whereas they may vary by up to 25 wavenumbers for dried cells. Thus, unlike for dried or fixed samples, no correction for band distortion and frequency shifts are necessary in the pre-processing stages of data manipulation.

Furthermore, we would also like to show that FTIR microspectroscopy is capable of differentiating live HeLa cells exposed to chemotherapeutical agents from untreated cells. Figure 6
shows the scores plot and the corresponding loading vector illustrating separation based on spectral variance between the cells untreated and the cells exposed to culture medium dosed with cyclophosphamide monohydrate, collected via image mode and pre-processed using NA-PC reconstruction. Cyclophosphamide monohydrate is an alkylating agent used in the treatment of various cancers.\textsuperscript{26, 27} This type of drug typically acts on DNA at the 7\textsuperscript{th} position nitrogen in guanine bases.\textsuperscript{16-18}

Figure 6, PC 2 demonstrates that most of the spectral variance between the untreated and drug exposed cells is occurring in the protein and low wavenumber regions. Specifically there are observable differences at the amide I position and the \( \text{PO}_2^- \) symmetric stretch vibration, as well as other minor changes in the low wavenumber region. These results indicate possible variations in the DNA, which are consistent with what is known about the mode of action for cyclophosphamide monohydrate. This drug typically targets the guanine bases in DNA in an effort to interrupt and halt cell function in proliferating diseased cells by causing inter- and intra- strand cross-links as well as other DNA mutations.\textsuperscript{16-18, 26, 27}

This experiment determined that it was possible to detect small spectral drug induced changes on live cells using the setup described, similar to previous experiments observing spectral changes based on environmental perturbations applied to the chamber.\textsuperscript{11} These cyclophosphamide results are related to an experiment that will be discussed in more detail in a future publication submission and were included here as an example to demonstrate the possible role of this technique as a tool in the development of cancer treatment plans.

**Conclusions**

The use of newly implemented data processing routines have improved our methodology for data acquisition by allowing cellular spectra to be collected as images, which has shown to be significantly faster than previous efforts via point mode. The ability to collect data at a faster rate allows more data to be collected and results to be more statistically meaningful. NA-PC reconstruction allows noise extracted from the positions lacking cells to be used to subtract noise polluting each cellular spectrum. This is noteworthy because spectral images require collecting ‘unoccupied’ space (tissue voids or pure solvent), which was previously thought to be a waste of time and storage space. We have shown that data collected from the voids serve a valuable purpose and permit computation of a ‘noise matrix’ for NA-PC reconstruction. We have demon-
strated that with the use of NA-PC reconstruction in conjunction with imaging data acquisition and spectral reconstruction via PapMap, data with improved S/N ratios can be obtained in a shorter collection time and with fewer manual operations (cell and aperture selection). Moreover, the absence of dispersive artifacts negates any necessity to perform correction algorithms. Finally, we conclude that by avoiding eigenvectors in the PC’s representative of atmospheric water, chemometric analysis such as PCA can be performed to demonstrate spectral variance within live cellular data. Live cell research is still in an early stage of development but with techniques like the one illustrated here, this methodology should eventually provide more desirable modes of treatment for patients in their diagnoses and treatments for disease.

Acknowledgment
Support of this research from grant CA 09034 is gratefully acknowledged.
References


\[ \text{noise} = \frac{\sum |A_i - \bar{A}|}{n} \]

**Equation 1:**

The noise used for S/N calculations was between 2000-2120 cm\(^{-1}\). Where, \(A_i\) represents the absorbance difference between each consecutive position along the wavenumber region and \(\bar{A}\), the mean of all \(A_i\). \(n\) represents the total number of data points used.
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**Table 1:**
S/N values for 10 individual cell spectra chosen at random from each of the three methods discussed: point mode, image mode before and after NA-PC reconstruction. At the bottom of each column is the average S/N for each of those methods.
Figure 1
Design of the live cell chamber used for IR-MS experiments. (A) Plexiglass stage plate. (B) Aluminum bottom plate which accommodates flow of culture medium to the window. (C) Top plate holds two separate heating cartridges. (D, not drawn to scale) depicts the two CaF$_2$ windows that form the cell chamber as viewed from above (top) and from the side (bottom).
Figure 2:
Pseudo color maps representing the noise areas (A), and the regions occupied by cells (B). Below each, examples of pixel spectra at positions indicated by the red arrows are shown.
Figure 3:
Examples of spectra collected via image mode (8 co-added interferograms per pixel) prior to (A) and after (B) NA-PC reconstruction, and of a point mode spectrum collected at 128 interferograms per pixel (C).
Figure 4:
Raw (A) and processed (B) data collected repeatedly from identical and different 1 mm x 1 mm sample spots. The PCA scores plot (C) demonstrates there is no spectral variance between individual image areas scanned on the same sample when excluding PC’s representative of atmospheric water vapor. The schematic (D) demonstrates where the consecutively scanned image areas were located in relation to one another. See text for details.
Figure 5:
Raw absorbance data (A) of amide I region of cells, indicating the absence of dispersive line shapes often observed at the edges of cells. Frequency map (B), where spectral maxima coincide at 1652 cm$^{-1}$. 
Figure 6:
Scores plot and loading vector 2 for live cells exposed to cyclophosphamide monohydrate and untreated live cells