Diagnosing Changes in Cells Using FTIR Microspectroscopy

Jing Guo
Georgia State University

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DIAGNOSING CHANGES IN CELLS USING FTIR MICROSPECTROSCOPY

by

JING GUO

Under the Direction of Dr. Gary Hastings

ABSTRACT

Fourier transform infrared (FTIR) microscopy has shown promise as an analytical tool for detecting changes in cells and tissues, such as those due to viral infection, apoptosis induction or malignancy. In many cases, diagnosis via FTIR microscopy can be undertaken on a timescale shorter than that required for other physical or histological techniques.

In this work we have used FTIR microscopy to study Vero cells that have been infected with herpes simplex virus (type I) and adenovirus. We have studied cellular samples at various time intervals following exposure to the virus. Several spectral regions were identified that allow discrimination between infected and uninfected Vero cell samples at 24 hours post exposure to both HSV1 and adenovirus. Spectral features were also identified that could be used to discriminate infected cells within 2-6 hours after exposure to both viruses. FTIR microscopy is
therefore a useful tool for following the kinetics of viral infection in the 2-24 hours time range, at least at the levels of infection used in this study.

In a second type of study, FTIR microscopy was used to study apoptosis induction in acute lymphoblastic leukemia T-cells. Apoptosis was induced in T-cells in three different ways. We show that FTIR microscopy can be used to distinguish T-cells in the early stages of apoptosis from normal cells. We also provide data that may suggest that FTIR microscopy can distinguish cells that have undergone apoptosis via different pathways.

For most of the FTIR microscopic studies on cellular samples we have focused on the collection of spectral data in the 1500-800 cm\(^{-1}\) region. Spectra were collected for control cells and variously treated cells. The two sets of cells were then analyzed statistically using: 1) pairwise comparison, 2) logistic regression, 3) partial least square regression, 4) principle component fed linear discriminant analysis and 5) hierarchical cluster analysis. The statistical analyses rigorously quantify to what extent treated and untreated cells can be distinguished. Since different statistical methods give differing results for the same data, it is important the right statistical method should be applied. The basis for these differences is discussed.

INDEX WORDS: FTIR Microspectroscopy, Vero cells, Herpes simplex virus, T-cells, Apoptosis.
DIAGNOSING CHANGES IN CELLS USING FTIR MICROSCOPY

by

JING GUO

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

in the College of Arts and Sciences

Georgia State University

2011
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May 2011
DEDICATION

I would like to dedicate this dissertation to my dearest husband Joseph R. Eggen and my greatest parents: Xiangzhi Wu and Qiang Guo.
ACKNOWLEDGEMENTS

First and foremost, I would sincerely like to express my deepest appreciation to my advisor Dr. Gary Hastings, who gave me the opportunity to work in the exciting field of biophysics. He has been a constant source of encouragement and support and guided me effectively throughout the whole work. I sincerely thank him for supporting me through the completion of my PhD dissertation.

I would also like to thank Dr. Yu-Sheng Hsu for introducing me to and guiding me through the world of statistics. His insights and his patience when answering my many questions were very integral to completing my data analysis.

I would also like to thank Dr. Julia Hilliard for enlightening me as to the overarching goals of the research and patiently guiding me through the biological nature of the project.

I would also like to take this opportunity to thank all my remaining committee members at Georgia State University, who extended full-fledged support and guided me to write this thesis in better ways than I could imagine on my own.

I would like to thank Dr. Ruili Wang, who has been an invaluable guide during the project period. Her skills in FTIR related software manipulation are greatly appreciated. I would like to thank all my project members, Dr. Pinhas Fuchs, Dr. John Ward, Chadi Filfili, Peter Krug, Irina Patrusheva, Nina Beato, Monique Wilson, Tang Tian, Luo Shan, and Jenny Jeyarajah, who extended their support and guidance from the time I joined the group. I would also like to thank all my group members, Sreeja Parameswaran, Hari Prasad Lamichhane, and Nan Zhao, who extended my knowledge about FTIR field. Each of these members has given his or her valuable assistant in the completion of this dissertation.
Last but not the least I thank my family and friends who have extended unflinching support and encouragement during my enjoyable academic time at Georgia State University.
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   Viral infection of cells in culture detected using infrared microscopy.
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<th>Description</th>
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<tr>
<td>A3 T-cells</td>
<td>cells that are a subclone of the Jurkat T-cell line</td>
</tr>
<tr>
<td>ALL</td>
<td>acute lymphoblastic leukemia</td>
</tr>
<tr>
<td>AF</td>
<td>anti-Fas</td>
</tr>
<tr>
<td>AV</td>
<td>annexin V</td>
</tr>
<tr>
<td>AV-FITC</td>
<td>AV-fluorescein isothiocyanate</td>
</tr>
<tr>
<td>AUC</td>
<td>area under the ROC curve</td>
</tr>
<tr>
<td>BT</td>
<td>bootstrap</td>
</tr>
<tr>
<td>CV</td>
<td>cross validation</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier transform infrared</td>
</tr>
<tr>
<td>HCA</td>
<td>Hierarchical cluster analysis</td>
</tr>
<tr>
<td>HPE</td>
<td>hours post exposure</td>
</tr>
<tr>
<td>HSV1</td>
<td>herpes simplex type I</td>
</tr>
<tr>
<td>LOOCV</td>
<td>leave one out cross validation</td>
</tr>
<tr>
<td>LDA</td>
<td>linear discriminant analysis</td>
</tr>
<tr>
<td>PLSR</td>
<td>partial least squares regression</td>
</tr>
<tr>
<td>PCA</td>
<td>principle component analysis</td>
</tr>
<tr>
<td>PS</td>
<td>phosphatidylserine</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>ROC</td>
<td>receiver operating characteristic</td>
</tr>
<tr>
<td>Vero cell</td>
<td>African green monkey kidney cell</td>
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</table>
1 INTRODUCTION

1.1 Background and Objective of the Project

Viruses are responsible for a variety of (sometimes severe or even fatal) infections in humans and animals. Recently, a great deal of research has focused on the use of different analytical techniques for the study of viral infection of cells. Two viruses of considerable importance as far as humans are concerned are herpes simplex virus type 1 (HSV1) and Adenovirus (ADV). HSV1 may infect the skin, eyes, genitals, lungs, and the brain [1-3], while ADV infects tissues that line the respiratory tract. Fourier transform infrared (FTIR) microscopy has been used for preliminary studies of cells infected with HSV1 and other herpes type viruses [4], (FTIR) microscopy has also been used for study of cells infected with other types of viruses [5], as well as for the study of bacterial and fungal infections [6], malignant cells [7] and strains of yeast cells used in wine making [8].

HSV1 is a complex virus that is difficult to distinguish from other possible infections caused by bacteria or fungi, particularly during the early stages of the infection. Isolation of the virus in cell culture and immunoassays are the main methods for the detection of herpes virus infections [9]. Such methods are time-consuming and relatively expensive, and an alternative faster approach is desirable [10]. Detection of viral infections at an early stage is often critical, as patient survival and quality of life may depend on it. On this sense early diagnosis of disease is usually seen as necessary in improving patient outcomes. Here we have developed the use of FTIR microscopy as a tool for identification of early biochemical changes within viral infected cells, with the goal of establishing the limits of applicability of the technique.
Cancer kills millions of people annually [11]. Morphological and histomorphological diagnosis methods for cancer classification are well established, and have remained relatively unchanged over the last several decades. Unfortunately, many of the methods for diagnosis and classification are time consuming and quite subjective, with very unsatisfactory levels of inter- and intra-observer discrepancy [12, 13]. New novel approaches for histological recognition are necessary to decrease subjectivity and provide new tools to investigate cancer in more detail.

FTIR microscopy has been shown to be a useful tool for the detection, identification and characterization of the molecular components of biological processes in cells, such as those responsible for the dynamic properties of cancer progression [14]. As the first step in the development of a robust analytical technique for use in cancer detection and diagnosis, we have used FTIR microscopy to study apoptosis induction in acute lymphoblastic leukemia T-cell. The eventual goal of this research is to apply it to the study of cells treated with drugs that may induce apoptosis in malignant cells. In this way it may be possible to assess the efficacy of new apoptosis-inducing drugs for different cell lines. Eventually it may be possible to test a priori how different patients may respond to new drugs actually administering the drugs.

FTIR microscopy is a potentially rapid and noninvasive technique for the diagnosis and detection of cells in different states. It can therefore have application in many areas, including disease detection, response of cells to drugs or other environmental stresses or genetic modifications. FTIR microscopy may be of use, especially, when little or nothing is known about an observed phenomenon. FTIR microscopy may provide a first hint that may be used to chart a path for further investigation.
1.2 Fourier Transform Infrared (FTIR) Spectroscopy

1.2.1 Infrared (IR) Spectroscopy

Infrared (IR) spectroscopy has long been recognized as being a valuable tool for identifying the structure and dynamics of materials, and it has been extensively employed over the years to investigate biological cells[15, 16]. In IR spectroscopy observed absorption peaks were assigned to different vibration states of molecules. However, in the spectra of biological cells molecular species can affect the absorption peaks. In addition, many other variables such as scattering or dispersion can also modify the peaks [17, 18]. The assignment of vibration peaks of the molecules is very difficult. The usual approach is to repeat the experiment many times, followed by statistical based analysis of the IR spectrum of cells [8].

IR spectrum is formed by measuring the wavelength dependence of the absorbance of IR light passing through a sample. The sample absorbs light at particular frequencies, while others are transmitted. Depending on the frequency, the IR spectrum is divided into three regions: near IR (NIR) region (above 4000 cm\(^{-1}\)), mid IR (MIR) region (4000-400 cm\(^{-1}\)) and far IR (FIR) region (400-10 cm\(^{-1}\)). The NIR region contains information about changes in vibrational level, overtones and some low energy electron transactions. The MIR region deals with changes in fundamental vibrational levels of molecules where as far IR gives information about rotational energy level changes. The FIR region provides useful information to structural studies such as conformation and lattice dynamics of samples which involve heavy atoms [19].

In “IR Spectroscopy: An Introduction”, the authors explain the usefulness of FTIR as follows: “Like a fingerprint of a person, the IR spectrum is highly characteristic for a substance and can be used for identifying it. The high specificity is based on the good reproducibility with which the coordinates of the absorption maxima (generally, wavenumber and transmittance) can
be measured.” [20]. Different molecular bonds absorb at different frequencies, so chemical processes can be observed by looking for different absorptions. Stretching and bending modes of vibrations of most molecules lie in the MIR region.

### 1.2.2 IR Absorbance

Infrared spectroscopy is the study of the interaction between infrared light and matter. Infrared radiation causes the molecular bonds in a species to vibrate with characteristic frequencies. The functional groups, or common molecular bonds, vibrate within the same frequency range regardless of the molecule they are present in. This property is utilized in infrared spectroscopy to identify these functional groups within a broad range of molecules. An infrared spectrum is a plot of intensity versus wavenumber; higher wavenumber values traditionally being on the left, and lower ones on the right. Wavenumbers are defined below (Sec. 1.2.3)

Normally absorbance or transmittance plots are obtained, in which the peaks point upwards or downwards respectively, showing the wavenumbers at which either process occurred. Apart from the qualitative information obtained from an infrared spectrum, concentration of the sample can also be estimated according to the Beer Lambert law as follows[18];

\[ A = \varepsilon lc \]  

(1.2.2.1)

Where \( A \) = absorbance; \( \varepsilon \) = molar absorptivity; \( l \) = path length; \( c \) = concentration;

The intensity of the absorption depends on changes in the dipole moment of the molecule. The dipole moment arises due to the atoms on either side of a bond tending to attract the electron forming the bond by different amounts. This can be thought of as creating small amounts of excess charge on the atoms, positive on one, negative on the other. The electric field
of a passing photon causes opposing forces on the two ends of the electric dipole, and induces an oscillation.[21]

Historically, many spectral analyses have been performed on known substances. By examining and comparing the results, some of the bands produced on a typical absorbance spectrum can be qualitatively assigned to the motion of particular bonds in the substance being studied. Molecular vibrations can take different forms, such as symmetric and asymmetric stretching, bending in and out of the plane of the molecule, and rocking.[22]

1.2.3 IR Spectra

An IR spectrum is usually expressed in terms of wavenumber, rather than wavelength. Wavenumber is often given the symbol $\tilde{\nu}$, and is calculated as the reciprocal of wavelength of IR light, measured in cm$^{-1}$, shown in equation 1.2.3.1.

$$\tilde{\nu} = \frac{1}{\lambda}$$

(1.2.3.1)

By examining the intensity of light passing through the sample at each wavenumber, a transmission spectrum can be produced, according to equation 1.1.3.2. This is a dimensionless quantity.

$$T_{\nu} = \frac{I}{I_0}$$

(1.2.3.2)

The absorbance A (also called optical density) is defined as:

$$A = -\log_{10}(I_0/I) = -\ln T$$

(1.2.3.3)

$I$ represents the light intensity after passing through the sample, and $I_0$ represents the intensity without passing through the sample. In practice, $I_0$ must be taken in a separate step as a reference spectrum with no sample present. An FTIR absorbance spectrum for vero cells is
shown in Figure 1-1. Wavenumber range is from 2000 – 800 cm\(^{-1}\). Y axis represents the absorbance.

![FTIR absorbance spectrum for vero cell.](image)

**Figure 1-1** An FTIR absorbance spectrum for vero cell.

### 1.3 FTIR Instrumentation

FTIR spectrometers are widely used. In the present study a Varian 7000 FTIR spectrometer equipped with an Mercury-cadmium-telluride (MCT) detector was used for recording IR spectra of samples mounted on Zinc Selenide (ZnSe) Windows. The fundamental part of the instrument is the so-called Michelson interferometer [23].
The Michelson interferometer consists of an IR source, a beam splitter, two mirrors, one of which scans, a reflecting mirror, and the detector. A sketch is shown in figure 1.2. The median ray is shown by the solid line, and the extremes of the collimated beam are shown by the dashed line. The infrared radiation, emitted by a broadband source, enters the interferometer and is split into two beams of equal intensity. Both beams are reflected back by the mirrors to the beam splitter. Depending on the movable mirror’s relative displacement, the beams at each cm$^{-1}$ interfere constructively or destructively. The interferogram, which is produced by the sum of the interferences, has its maximum at x=0, because all wavelengths interact constructively, giving rise to a “center burst”. The detected signal is amplified, processed and recorded. Finally, the interferogram is Fourier transformed to get a transmittance spectrum.

Figure 1-2 The sketch of a Michelson interferometer.
Figure 1-3 Interferograms measured using FTIR spectrometer.

Figure 1-3 shows interferograms measured using an unfiltered globar source (B) and a source with a 2000-1000 cm\(^{-1}\) filter in the light beam (A). Interferograms have been normalized. The x-axis represents the position of the moving mirror, which means the data point is proportional to the difference in optical path length of the two interfering beams.[25]

FTIR spectrometers have two major advantages compared to dispersive instruments. The first is called Jacquinot advantage [26], which is related to higher throughput of radiation in an FT-IR instrument. This is because there are no slits to restrict the intensity of radiation striking the detector in an FTIR instrument.
The second advantage is called Fellgett’s advantage, or multiplex advantage [26], denoting the fact that all the wavenumbers of radiation strike the detector at the same time, meaning a faster acquisition time. In a dispersive instrument only specific wavenumbers are let through the slit to strike the detector, slowing down the acquisition process.

Fellgett’s advantage relates to the fact that the signal to noise ratio (SNR) is proportional to the square root of the number of scans taken for a particular sample [19]. So for a similar time period to obtain a sample spectra, an FT-IR instrument has a significant advantage vs. dispersive instrument since it can take more scans within the same length of time, giving a better SNR [19]. Besides simplicity, reliability, speed (Fellgett’s advantage), and high energy throughput (Jacquinot advantage)[26], a FTIR spectrometer also possesses accuracy in frequency determination (Connes’s advantage).

1.3.1 The Light Source

The silicon carbide (SiC) globar source is a simple and relatively inexpensive IR light source covering the near to mid infrared emission region that is widely used in FTIR spectrometers.

1.3.2 The Beam Splitter

The beam splitter is simply a piece of semi-reflective material, usually a thin film sandwiched between two pieces of IR-transparent material. The beam splitter equally splits the IR beam.

Potassium Bromide (KBr) is commonly used for mid-IR beam splitters for FTIR spectrophotometers. The efficiency of a KBr beam splitter greatly increases in the MIR region. Therefore, a KBr beam splitter can be used down to 800 cm$^{-1}$. 
1.3.3 The Detector

Mercury cadmium telluride (MCT) detectors are highly sensitive in the 6000-700 cm\(^{-1}\) region. Experiments reported here a liquid nitrogen cooled 0.5x0.5 mm MCT detector was used.

1.3.4 Filter and Windows

In our experiments we are interested in the 2000-700 cm\(^{-1}\) region. A filter is necessary to cut off the unwanted radiation. A 2000-700 cm\(^{-1}\) band-pass filter was placed in front of the MCT detector to limit the spectral region. Gives better SNR, the spatial extent of the IR light impinging on the samples was limited by variable apertures to an area of ~0.1x0.1 mm. 13 mm diameter x 1mm thick zinc selenide (ZnSe) infrared transmission windows, transparent in 5000-500 cm\(^{-1}\), were used in placing liquid sample cells used with infrared and spectrophotometers.

Figure 1-4 shows a FTIR spectrometer layout[27]. Infrared beam is emitted from a glowing black-body source (glow bar). This beam passes through an aperture which controls the amount of light that passes through the sample and to the detector. The beam enters the Michelson interferometer as we described previously. The resulting interferogram signal is transmitted through (or reflected off of) the surface of the sample. After the absorption takes place in the sample, the rest of the beam passes to the detector for measurement. Then the measured signal is Fourier transformed in the computer. The final infrared spectrum is then ready for further manipulation and analysis[27].
1.4 Statistical Treatment of Experimental Spectra

Many different studies have investigated the possibility of developing FTIR microscopy as a diagnostic method. Salman et al. (2002) have applied Cluster analysis to show that FTIR microscopic signatures can be used to differentiate normal cells from cells infected with herpes. According to Alam et al. (2004), activated murine (mouse) macrophage cells can be distinguished from live cells before activation using Principal Components Analysis (PCA) coupled with Linear Discriminate Analysis (LDA) and K-Nearest Neighbor (K-NN) models. Burattini et al. (2008) have applied two multivariate statistical analysis methods - Hierarchical Cluster Analysis (HCA) and PCA - to compare the spectral behavior of *S. cerevisiae* in model wine medium and base wine, before and after 5 days of autolysis. Babrah et al. (2009) also applied the PCA-fed LDA model to select biomolecular signatures to discriminate the leukaemia cell lines be-
tween T-cell lymphoma, B-cell lymphoid, and myeloid leukaemia. It is well known that Partial Least Square Regression (PLSR) has already become a very popular method in food science; it is also starting to develop in a novel detection scheme for viral infection using FTIR and cell culture [28]. In the following sections, the different statistical methods, along with their strengths and weakness, will be described.

1.4.1 Hierarchical Cluster Analysis

The first and most intuitive method for comparing two sets of spectra is to calculate the difference. However, the cumulative effects of the various sources of uncertainty in the experiment result in a low consistency between spectra. Thus, it is difficult to identify spectral features that correlate with changes in the sample by simply comparing difference spectra. However, the simplest method - repeating the experiment many times in order to average-out the errors - would prove too time-consuming. Therefore, we must choose a statistical analysis method which is both quick and provides the optimum sensitivity/specificity, in order to reliably correlate changes in the sample spectra with changes in the cellular state.

Hierarchical cluster analysis (HCA) is a commonly used statistical method for clustering different groups. It starts with all of the observations (spectra) in one cluster and then proceeds to split (partition) them into smaller clusters according to the variation in the spectra data sets. First, a distance matrix is calculated which quantifies the similarity of the spectra. This matrix is symmetric and of size n-by-n, where n is the number of spectra. We choose D-Values (explained below) to obtain inter-spectral distances. Next, the two most similar spectra, defined as spectra with the smallest inter-spectral distance, are determined. These spectra are combined to form a new object (cluster). The spectral distances between all remaining spectra and the new object are then
recalculated using Ward's algorithm defined in the CytoSpec Program. A new search for the two
most similar objects (spectra or clusters) is then initiated. These objects are merged and again,
the distance values for the newly formed cluster are determined. This procedure is performed n-
1 times until only one cluster remains.

As mentioned above, for HCA the distance method we choose for our distance matrix
calculation is called D-Values, and is formula 1.4.1.1 shown below:

\[ d_{y1y2} = (1 - r_{y1y2}) \times 1000 \]  (1.4.1.1)

\[ r_{y1y2} \] is known as the Pearson’s correlation coefficient:

\[ r_{y1y2} = \frac{\sum_{i=1}^{p} y_{1i} y_{2i}}{\sqrt{(\sum_{i=1}^{p} y_{1i}^2 - p \times y_{1}^2) \times (\sum_{i=1}^{p} y_{2i}^2 - p \times y_{2}^2)}} \]  (1.4.1.2)

where \( p \) is the total number of absorbance values in the spectra, and \( y_{1i} \) and \( y_{2i} \) are the \( i^{th} \) ab-
sorbance values of spectrum 1 or 2, respectively.

Ward's algorithm is distinct from all other clustering methods. It uses an analysis of va-
riance approach to evaluate the distances between clusters. This method attempts to minimize the
Sum of Squares (SS) of any two (hypothetical) clusters that can be formed at each step. This me-
thod is regarded as very efficient in general; however, it tends to create clusters of small size.[29]

### 1.4.2 Principal Component Discriminant Analysis

The principal component analysis–linear discriminant analysis (PCA-LDA) method has been used for statistical analysis of FTIR spectra of cells [30, 31].

Principal Component Analysis (PCA) [32, 33] is used to reduce the dimensionality of the
data while retaining the variation present in the original dataset as described in Equation 1.4.2.1. Using PCA the first step is to standardize the data by subtracting the mean and dividing by the
standard deviation. Second, the covariance matrix is produced. Third, by computing the eigenvalues from the covariance matrix, standardized data can be mapped into a lower-dimensionality space. This is done by sorting the new eigenvectors and choosing the first few principle components so that allows the dimensionality reduction step to be accomplished.

\[
x = \begin{bmatrix}
    a_1 \\
    a_2 \\
    \vdots \\
    a_N
\end{bmatrix} \rightarrow \text{reduce dimensionality} \rightarrow y = \begin{bmatrix} b_1 \\
    b_2 \\
    \vdots \\
    b_K
\end{bmatrix} (K << N)
\]

where \( x \) is the original data matrix with \( N \) dimension, and \( y \) is the new data with \( K \) dimension after \( x \) is mapped into a lower-dimensionality space.

Linear discriminant analysis (LDA)[34] is used to determine which variables discriminate between two or more groups. Canonical analysis, which is the LDA method we used for this project, can perform a multiple group discriminant analysis. It determines an optimal combination of variables so that the first function provides the most overall discrimination between groups; the second provides second most, and so on. Moreover, the functions will be independent or orthogonal, that is, their contributions to the discrimination between groups will not overlap. Computationally, we performed a canonical correlation analysis that determined the successive functions and canonical roots (the term root refers to the eigenvalues that are associated with the respective canonical function).

### 1.4.3 Wilcoxon Signed-Rank Test

To find what frequencies in the spectra are most significant, we applied the Wilcoxon Signed-Rank Test (WSRT, one sample test) to spectra data. WSRT is a non-parametric test. Therefore, it is very robust to the influence of outliers (individual observations that stick out from the pattern) and it does not require assumptions about the form of the distribution of the
measurements[35]. Furthermore, using signed ranks instead of the real values may be an efficient method for discriminating between different groups rather than differentiating different means.

The procedure we used is as follows:

1. For each wavenumber (treated as a variable), the null hypothesis $H_0$ is that the mean of intra data $M_{\text{intra}}$ is equal to zero.

   \[ H_0: M_{\text{intra}} = 0 \]

2. Obtain WSRT statistics: the difference $d_i$ between each observation and the hypothesized mean $M_{\text{intra}}$,

   \[ d_i = x_i - \text{Medium}, \]

   where $x_i$ is the $i^{th}$ observation (spectral intensity for $i^{th}$ wavenumber).

3. Rank of the set \{ | $d_i$ | : $i = 1, \ldots, n$ \} and name them, $Y_i$, $i = 1, \ldots, n$.

4. Define $R_i = (\text{sign } x_i) Y_i$. Then the Wilcoxon rank statistic $W$ is defined to be the sum of all positive $R_i$.

5. Under the null hypothesis $H_0$, the mean ($\mu$) and the standard deviation ($\sigma$) of $W$ is given as:

   \[ \mu = n(n +1) / 4, \]

   \[ \sigma = n(n +1)(2n +1) / 24, \]

   where $n$ is the sample size.

6. Under the normal approximation, the p-value is given by $P(Z > |z|)$

   \[ z = (W - \mu) / \sigma \]

   We can select the significant wavenumbers by the p-value among all 182 wavenumbers.

7. The Bonferroni method[36] is used to calculate the adjusted probability of type I error (false positive) in the multiple variables case. Therefore, the critical value for p-value should be around $0.05 / (2*182) = 0.0002$. For all 182 variables, only the one whose p-value of WSRT is smaller than or close to 0.0002 should be selected as significant variables for the next step of the analysis.
1.4.4 Partial Least Square Regression and Logistic Regression

In chapters 2 to 5 Partial Least Square Regression (PLSR) or Logistic Regression (LR) is used to diagnose if cells are infected or not, or whether cells have undergone apoptosis.

Variables can be categorized into observable and unobservable groups. MacDonald (1996) stressed that a variable can be called observable “if and only if its value can be obtained by means of a real-world sampling experiment”. Therefore, any variable that does not correspond directly to anything observable must be considered as unobservable. For example, Wold (1993) pointed out that the effects and properties of molecules, genes, viruses and bacteria are usually observed only indirectly, i.e. unobservable variables.

Also, as presented in Equation 1.4.4.1, PLSR requires two sets of variables: independent and dependent. Absorbances at significant wavenumbers from the WSRT were treated as independent variables. The dependent variables (Y, also response variables) were coded as 0 and 1 to represent two samples we were trying to discriminate.

PLSR is a method for constructing predictive models when the independent variables (absorbances at different wavenumbers) are highly correlated. It is particularly useful when the matrix of predictors has more variables than observations. The overall goal is to use the predictors to predict the responses in the population. First, PLSR estimates the weight relations based on the unobservable variables. Then case values are calculated, based on a weighted average of its indicators, using the weight relations as an input. Second, these case values are used in a set of regression equations (Equation.1.4.4.1) to determine the parameters for the structural relations.[37] In statistics, case value is the estimated value for each latent variable in each data set. Latent variables, called predictors, or factors, (as opposed to observable variables), are variables that are not directly observed but are rather inferred (through a mathematical model) from other
variables that are observed (directly measured). A PLSR model can be expressed as the projection to latent structure.

\[ Y = f_1U_1 + f_2U_2 + \cdots + f_nU_n + E, \]  

(1.4.4.1)

where \( U_i, i = 1, \cdots, n \) are factors, and \( f_i, i = 1, \cdots, n \) are the coefficients of them, and also called discriminators. PLSR uses variable combinations \( U_1 \) (the first factor), \( U_2 \) (the second factor), \( \cdots \), \( U_n \) (the \( n \)th factor), such that

\[
\begin{align*}
\max_{\|\boldsymbol{x}\| = 1} \quad & \text{corr}^2 (Y, U_i) \text{Var} (U_i) \\
\text{s.t.} \quad & x_i \alpha = 0, i = 1, \cdots, i - 1
\end{align*}
\]  

(1.4.4.2)

where \( U_i = X\alpha_i, i = 1, \cdots, n \), \( S \) is the sample covariance matrix, \( X \) is a \( I \times J \) matrix which contains all the values of \( J \) predictor variables collected on \( I \) observations, and \( Y \) is a \( 1 \times 1 \) matrix storing the \( I \) observations described by the dependent variable. The conditions

\[ \alpha_i^T S\alpha_i = 0, i = 1, \cdots, i - 1 \]

ensure that \( U_i = X\alpha_i \) is uncorrelated with all the previous linear combinations \( U_i = X\alpha_i, i = 1, \cdots, i - 1 \) [38].

PLSR finds combinations of the predictors that have a large variance and highly correlated with the response variables. Similar to PCA, PLSR produces a linear regression model by projecting the dependent variables and the independent variables to a new space. Therefore, we can find fundamental relations between two sets of variables.

Another popular statistical method is Logistic regression (LR). LR is also a type of predictive model that can be used when the target variable is binary. The LR model yields the probability of occurrence of an event by fitting data to a logistic curve. In other words, the correlation between the discriminators and response variables is not a linear function in LR. Instead, LR
finds a linear combination of \( x_i \) (the input data), which is the logit transformation of the probability of success \( g \), i.e.

\[
\log \, \text{it} \ g = \log \left( \frac{g}{1-g} \right) = \alpha + \beta_1 x_1 + \beta_2 x_2 + \cdots + \beta_n x_n
\]  

(1.4.4.3)

or equivalently,

\[
g = P(Y = 1 | X) = \frac{e^{(\alpha + \beta_1 x_1 + \beta_2 x_2 + \cdots + \beta_n x_n)}}{1 + e^{(\alpha + \beta_1 x_1 + \beta_2 x_2 + \cdots + \beta_n x_n)}}
\]

(1.4.4.4)

where \( \alpha \) is the constant of the equation, \( P(Y=1|X) \) is the probability function when \( Y=1 \) for any \( X \), \( Y \) is outcome variable, \( X \) is the independent variable, and \( \beta_i, i = 1, \cdots, n \) are the coefficients of the predictor variables.

There are many ways to select the number of discriminators included in the PLSR or LR models. We simply use the number of discriminators which count a certain percent of the total variation (say 80, 90, 95%), which can be limited by certain P-values. Then we can evaluate the model diagnostic performance by deriving area under the ROC curve (AUC) sensitivity and specificity as mentioned later in this chapter.

### 1.4.5 Area Under the Curve, Sensitivity and Specificity

After building a model, we then need to evaluate its diagnostic performance, namely the ability to correctly classify two categories. Usually we can use sensitivity and specificity, and the area under the Receiver Operating Characteristic (ROC) curve to make the evaluation. In this
project we used two methods to calculate sensitivity, specificity and the area under the ROC (AUC).

For the first method, since the discriminators from the PLSR have approximate normal
distribution, we can estimate AUC, sensitivity and specificity as follows.
Let $X_1$ and $X_2$ be the discriminators for inner and intra-difference, respectively.

\[ AUC = P\left(X_2 > X_1\right) = P(X_2 - X_1 > 0) \]  \hspace{1cm} (1.4.5.1)

where $P$ is probability, $E(X_2 - X_1) = \mu_2 - \mu_1$, and $\text{Var}(X_2 - X_1) = \sigma_1^2 + \sigma_2^2$, where $E$ is the expected value and Var is the variance.

\[ Sensitivity = P(X_1 \leq C) = P\left(\frac{X_1 - \mu_1}{\sigma_1} \leq \frac{C - \mu_2}{\sigma_2}\right) = \phi\left(\frac{C - \mu_2}{\sigma_2}\right) = 1 - \alpha \]  \hspace{1cm} (1.4.5.2)

\[ C = \phi^{-1}(1-\alpha)\sigma_2 + \mu_2 \]  \hspace{1cm} (1.4.5.3)

Therefore

\[ Specificity = P(X_2 \geq C) = P\left(\frac{X_2 - \mu_2}{\sigma_2} \geq \frac{C - \mu_2}{\sigma_2}\right) = \phi\left(-\frac{C - \mu_2}{\sigma_2}\right) = \frac{\mu_2 - \mu_1 - \sigma_2\phi^{-1}(1-\alpha)}{\sigma_2} \]  \hspace{1cm} (1.3.5.4)

and

\[ AUC = P(X_1 \leq X_2) = P\left(\frac{(X_1 - X_2) - (\mu_1 - \mu_2)}{\sqrt{\sigma_1^2 + \sigma_2^2}} \leq \frac{\mu_2 - \mu_1}{\sqrt{\sigma_1^2 + \sigma_2^2}}\right) = \phi\left(\frac{\mu_2 - \mu_1}{\sqrt{\sigma_1^2 + \sigma_2^2}}\right) \]  \hspace{1cm} (1.3.5.5)

where $\Phi$ is the distribution function of the standard normal distribution. [39]

The estimated AUC, sensitivity and specificity can be obtained by replacing $\mu_1, \mu_2, \sigma_1, \sigma_2$ with the sample means and sample standard deviations.
The second method is the standard calculation for all three parameters. Sensitivity and specificity, as shown in Table 1-1, are closely related to the concepts of type I and type II errors. Sensitivity measures the proportion of correct identifications among actual positives, such as the probability of a positive test among patients with a particular disease; and the specificity measures the proportion of correct identifications among all negatives, such as the probability of a negative test among patients without said disease. [38]

The estimated sensitivity and specificity can be expressed as

\[
\text{Sensitivity} = \frac{a}{a+c} \quad (1.3.5.6)
\]

\[
\text{Specificity} = \frac{d}{b+d}, \quad (1.3.5.7)
\]

Where a, b, c, and d are defined in Table 1-1.

<table>
<thead>
<tr>
<th></th>
<th>Cells infected with virus</th>
<th>Cells without infection (mock)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test is positive(infected)</td>
<td>a</td>
<td>b</td>
</tr>
<tr>
<td>Test is negative(uninfected)</td>
<td>c</td>
<td>d</td>
</tr>
</tbody>
</table>

In the table above, a represents the true positives (infected samples correctly diagnosed as infected); b represents the false positives (uninfected samples incorrectly diagnosed as infected); which; c are the false negatives (infected samples incorrectly diagnosed as uninfected); d are the true negatives (uninfected samples correctly diagnosed as uninfected).
A complete description of classification is given by the area under the ROC curve (Figure 1-5), which is a plot of the sensitivity against 1-specificity for the different possible cut-off points of a diagnostic model. Each point on the ROC curve represents a sensitivity and specificity pair corresponding to a particular decision threshold. As shown in Figure 1-4, the blue line represents a ROC curve with high AUC and the green line represents a ROC curve when AUC equals 0.5. When the sensitivity increases, the corresponding specificity will decrease. If the objective is to choose an optimal cut-off point for the purpose of discrimination, one might select a cut-off point that maximizes both sensitivity and specificity. An area of 1 represents a high accuracy of discrimination, and an area of 0.5 represents very low accuracy. A rough guide for classi-
fying the accuracy of discrimination is the traditional academic point system. That is, Area Under the Curve between 0.90 and 1 represents excellent discrimination; AUC between 0.80 and 0.90 represents good discrimination; AUC between 0.70 and 0.80 represents fair discrimination; AUC between 0.60 and 0.70 represents poor discrimination; and AUC between 0.50 and 0.60 represents no discrimination.[38]

### 1.4.6 Bootstrap and Cross-validation

In statistics, the goodness of a procedure for given sample data is frequently over-rated, which means most model fitting procedures often yield results that are highly dependent on the input data set used to build the model. In order to estimate the precision of sample statistics, estimate the sampling error or validate the model, we can use bootstrap methods[40] by drawing randomly with replacement from a set of data, or cross-validation by using random subsets from the original data sets. We refer to this sampling error as “shrinkage”, which is defined as the difference between the specificities (or sensitivities, or AUC) of two data sets: the population and the observed sample.

Since the model derived from the observed sample could not represent the whole population, shrinkage becomes an important indicator for the future performance of this model in discriminating the unknown data set. In order to estimate the shrinkage, we calculated the difference of the specificities (or sensitivities, or AUC) between the model data sets and test data sets.

We employed cross-validation and bootstrap to resample the model and test datasets. Cross-validation, a method of estimating sampling error, can be used to assess the shrinkage of the AUC and specificities of the model we built. In K-fold cross-validation, the original sample is randomly divided into K subsets of approximately equal size. Of the K subsets, a single subset
is retained as the validation data, and the remaining K-1 subsets as a whole are used as training data which is used to build the model. The cross-validation process is then repeated K times, with each of the K subsets used exactly once as the validation data. The K results can then be averaged to produce a single estimate.

In chapter 2 to 5, we employed 2-fold cross-validation to examine the accuracy of the AUC and specificity found in the models. The original data for building the model were randomly divided into two equal subsets. AUC and specificities which correspond to the sensitivities 95%, 90% and 80% were calculated by validation data and training data respectively. We then obtained the shrinkages by subtracting AUC and specificities for validation data from the ones for training data. After we repeated the process n times, the averaged shrinkage of AUC or specificities were then subtracted from original sample estimates to obtain the final estimations from cross-validation.

2-fold cross-validation normally gives an over-estimated shrinkage due to the reduction of the sample size. Unlike 2-fold cross-validation, bootstrap method normally gives an underestimated shrinkage. Model datasets for bootstrap were calculated by constructing a number of resamples. Resampling entailed drawing randomly with replacement from a set of data points of the observed dataset, which were of equal size to the original observed datasets. Test datasets refers to the original observed dataset. By applying the same procedure as mentioned above, we obtained the AUC and specificities for the validation data and training data respectively. Repeating the whole procedure n times, we were then able to use the final averaged shrinkage to calculate the final estimations from bootstrap.

By averaging these final estimations from two methods, we can have a reasonable estimate of the shrinkages.
In the following chapters, I will discuss the various aspects and findings of my thesis. In Chapter Two I will describe the studies of cells infected with different viruses at 24 hours post exposure. Different statistical method will be applied to the same datasets, such as HCA, PLSR and LR. In Chapter Three, I will present the studies of viral infected cells at 8, 12 and 24 hours post exposure, as well as describe the statistical results from PC-LDA and PLSR. Similarly, in Chapter Four, I will describe how cells infected with viruses at 2, 4 and 6 hours post exposure were studied as in Chapter Three. For Chapter Five, apoptosis in Acute Lymphoblastic Leukemia T-Cells were studied under different stresses by using FTIR and different statistical analyses will be described.
2 STUDIES OF VIRAL INFECTED CELLS AT 24 HPE

2.1 Viral infection of cells in culture detected using infrared microscopy

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Summary

FTIR microscopy has been used to collect spectra for uninfected (mock) Vero cells, and cells that have been infected with herpes simplex virus type 1 (HSV-1) and human Adenovirus type 5 (Ad-5). Cells were infected at a multiplicity of infection of 10, and studied at 24 hours post exposure. The spectra for infected samples display many differences compared to spectra for uninfected samples.

To estimate how well spectra for uninfected and infected samples could be discriminated we used logistic and partial least squares regression methods. We show that spectra for HSV-1 and mock infected samples are well differentiated, and for a sensitivity of 95%, we calculate a specificity of 0.999 using partial least squares regression. Spectra for Ad-5 and mock infected samples are also well differentiated. We find that applying our regression models constructed with one data set to a new validating data set still gives very high levels of specificity for a given sensitivity.

Spectra for Ad-5 and HSV-1 infected samples are also differentiable. Applying our constructed regression models to new validating data, however, leads to a decrease in discrimination capability in this instance, however.

If one is simply interested in differentiating spectra associated with uninfected and infected cells, without distinguishing the type of infection, then we show that logistic regression models can break down whereas partial least squares regression models perform well.
Introduction

Infrared spectroscopy and microscopy of cells in culture is a rapidly growing area of research, the goal of which is the ability to spectrally distinguish between cells in different physiological states. In our lab one goal is to use infrared spectroscopy to develop robust methods that can rapidly and accurately differentiate between uninfected cells and cells that have been infected with viruses.

Rapid and accurate detection of viral infections is of importance not only in the realms of disease detection in humans, animals and plants, but also in the realms of homeland security where a rapid detection and diagnosis of airborne and environmental biological agents is desirable.

In our work we are particularly interested in viruses that infect humans, and here we report on the detection of spectral changes in African green monkey kidney cells (Vero cells) that occur as a result of infection with herpes simplex virus (type 1) or Adenovirus. In this sense the Vero cells act as sensors of viral infection, responding to the virus with morphological or biochemical changes that we detect spectroscopically. Herpes simplex virus (type 1) (HSV-1) is predominantly involved with infections of the skin and central nervous system, and is difficult to distinguish from other possible infections caused by bacteria or fungi, particularly at the early stages of the infection. Isolation of the virus in cell culture and immunoassays are the main methods for the detection of herpes virus infections. Such methods are time-consuming and relatively expensive, and an alternative faster approach is desirable. Scientists have begun exploring the application of FTIR spectroscopy for detection of HSV-1 and other herpetic infections, as well as applications to other types of viral, bacterial and fungal infections. However, we believe
that much of this previous work has oversimplified the true capabilities of FTIR spectroscopy as a method for detecting infections in cells (see below). In this paper we attempt to present a more rigorous approach to spectral detection of pathogens in cellular material.

One common mistake made when investigating the use of FTIR spectroscopy as a cellular monitor, is to specifically assign small changes in certain spectral regions to specific molecular changes in infected or diseased cells. For example, in FTIR spectra of cellular samples some investigators report that peaks at or near 1237 and 1082 cm\(^{-1}\) are unambiguously attributable to PO\(^2-\) asymmetric and symmetric stretching vibrations of nucleic acids. They then infer some change in cellular DNA due to infection. Such inferences are often problematic:

Firstly, directly related to the above example, it has been established that nuclear material is on the whole opaque to infrared (IR) radiation \(^{11, 12}\). Secondly, it has been established for cellular samples that any IR peak in virtually any spectral region is a composite that consists of underlying contributions from many cellular components \(^3\). To show that specific bands are associated with specific molecular groups, controlled experiments have to be undertaken in which the specific molecular species under consideration is modulated. We have recently undertaken such an experiment to establish which IR bands are specifically associated with cellular lipids. Such an analysis was accomplished by specifically removing most of the cellular lipids from the cellular material\(^2\).

Thirdly, we show below that spectra for cell samples can vary considerably depending on the location on the sample that the spectra are sampled. Even the spectra obtained for presumably identical single cells can vary considerably \(^{13}\). The spectral differences are due to slightly different light scattering characteristics of the cells, which are a function of the cell shape, size and sub-cellular composition \(^{11, 13}\). Therefore, it is possible that cells that are infected may undergo
structural alterations that can change the infrared light scattering characteristics of the cellular material, compared to uninfected cellular material. Such variation in spectra due to sampling slightly different (structurally) cellular suspensions is virtually never considered as the possible origin of observed spectral changes between cells in different states. Furthermore, baseline correcting IR spectra prior to analysis may serve to mask issues that may be associated with changing IR light scattering characteristic of cells. Given these considerations, we try to quantify the extent of spectral variation due to sampling different areas of a cellular suspension, and we then try to assess whether spectral changes that may be associated with cellular infection are in fact larger than the intrinsic spectral variation due to sampling. Even after such an analysis it is still difficult to unambiguously specify that the spectral changes observed in infected cells are in fact due to changes in cellular composition caused by the infection. That is, it is difficult to rule out that spectral changes may be caused by different scattering characteristics of uninfected and infected cellular suspensions (see below). However, one could argue that a change in the scattering characteristic of cellular suspensions due to infection is still a useful diagnostic measure of infection.

In this paper we use infrared spectra of dried suspensions of African green monkey kidney cells (Vero cells) to report on the infection status of the cells. The spectral changes due to infection are small, especially when compared to the spectral variation that results merely from sampling the suspension at various locations on the dried suspensions (see below). It is almost impossible to infer viral infection of cells simply by collecting a single spectrum from some location on the cellular suspension. However, by sampling (collecting spectra) at many locations on the suspension, and applying advanced statistical methods to the analysis of all of the spectra obtained, we show that it is possible to distinguish with a high degree of accuracy, infected from
uninfected cellular material. We also show that with the use of such statistical methods, it is possible to distinguish different types of viral infections.

Experimental

Cell Culture and Preparation. African green monkey kidney cells (Vero, American Type Culture Collection #CCL-81) were grown at 37°C in DMEM (Mediatech, Manassas, VA) supplemented with 10% Fetal Bovine Serum (Atlanta Biologicals, Atlanta, GA), 100 units/ml penicillin, and 100 μg/ml streptomycin (Mediatech, Manassas, VA). Once confluent (generally within 24 hours of seeding), the Vero cell monolayers were infected with herpes simplex virus (type 1) HSV-1 or human Adenovirus-5 (Ad-5), at a multiplicity of infection (MOI) of 10 for 2 hours. Following this the growth medium (plus excess virus) was replaced with new growth medium. For mock infections, a normalized volume of uninfected cell lysate was incubated with the cells. At 24 hours post exposure (for the work described here) cells were scraped from the surface of the growth medium and centrifuged at 800 g for 1 minute. Cells were resuspended in 5 μl of PBS buffer containing calcium and magnesium. Several 0.5 μl drops were pipetted onto ZnSe windows and allowed to dry in a Class IIB biosafety cabinet. In the experiments reported here, cells were not subjected to any chemical fixation protocol (except air drying).

FTIR measurements: FTIR microscopy measurements in transmission mode were undertaken using a Varian 7000 FTIR spectrometer, coupled to a Varian UMA600 IR microscope (Stingray system). The microscope system contains only reflective optical elements consisting of on-axis, matched 15X Schwartzchild objective and condenser. The microscope is also equipped with a visible light source and a visible 640x480 CCD camera, allowing one to obtain visible microscopic images from the same region that is sampled in the IR experiment. In the experiments reported here a liquid nitrogen cooled 0.5x0.5 mm mercury cadmium telluride (MCT) detector
was used with high responsivity in the 6000-700 cm\(^{-1}\) region. The spatial extent of the IR light impinging on the samples was limited by variable apertures to an area of \(\sim 0.1\times0.1 \text{ mm}\) (see figure 1). A 2000-700 cm\(^{-1}\) band-pass filter was placed in front of the MCT detector to limit the spectral region. All measurements reported here were undertaken at 2 cm\(^{-1}\) resolution. In many cases the spectra we collected had amide I and II bands with absorption considerably above 1.4 (in optical density units). For this reason we consider only data collected in the 1500-800 cm\(^{-1}\) region, where all absorbance values are much lower (figure 2). No baseline correction algorithm was applied to any of the spectra collected.

In a typical experiment we usually pipette 5x0.5μL drops of cellular material onto a ZnSe window. These drops form a dried suspension with a diameter of \(\sim 1-2 \text{ mm}\). The ZnSe windows are placed in the sample compartment of the FTIR microscope, which is enclosed and continually purged with dry air. Roughly 60-100 spectra are collected at various locations over the five sample drops. Sixty four spectra are collected at each location and averaged. Locations on the dried sample are selected manually so as to ensure that cellular material covers the whole of the sampling aperture (figure 1).

Statistical Analysis. All statistical analysis of spectra reported here were undertaken using home-written code implemented within the SAS (Cary, NC) software programming environment. In this manuscript we have collected spectra for mock, HSV-1 and Ad-5 infected cells (at a multiplicity of infection (MOI) of 10 and at 24 hours post exposure, on two separate occasions. Data collected on the first occasion was used to construct a statistical model. Data collected on the second occasion was used to validate the constructed statistical model (see below).
Results

Figure 1 shows two visible images from a single air-dried cellular suspension of mock infected cells. Infrared light is only transmitted through the central “square” aperture. At each of the two different sampling locations spectra were collected. Usually we collected 60-100 spectra dispersed over 4-5 sample “drops”. Very similar images to those shown in figure 1 were obtained for HSV-1 and Ad-5 infected cellular suspensions.

Figure 2 shows the raw spectral data collected for mock and HSV-1 infected suspensions (A), and mock and Ad-5 infected suspensions (B). The spectra of mock infected samples are shown in black and the viral infected samples in red. For mock/HSV-1/Ad-5 infected samples spectra were collected at 69/79/94 locations on the cell sample “drops”, respectively.

Figures 2A and 2B show that there is considerable variation in the intensity of the spectra collected at various locations on the dried cellular suspensions. In figure 2A, the overall trend appears to be that the HSV-1 infected cells have decreased absorption compared to mock infected cells. This could be because the mock infected cellular suspensions are thicker than the HSV-1 infected suspensions, or because the concentration of cells is slightly higher for the mock infected suspensions. In either case we have consistently found that spectra for HSV-1 infected suspensions display decreased absorption relative to the spectra for mock infected suspensions. On this basis alone, it appears that HSV-1 infected cellular suspensions can be distinguished from mock infected suspensions (at MOI=10 and 24 hours post exposure). A similar but less pronounced trend in the spectra is also observed for Ad-5 infected cellular suspensions compared to mock infected cellular suspensions (figure 2B).

To eliminate effects due to sample thickness or cellular concentration, the spectra in figure 2 were standardized. A standardized spectrum is obtained by first mean centering the original
spectrum and then dividing by the standard deviation. The standardized spectra calculated from the raw spectral data in figure 2A/B are shown in figure 2C/D, respectively.

Figure 2C and D indicate that there are several spectral differences between mock infected and HSV-1 or Ad-5 infected suspensions. These features are more easily visualized in the averaged spectra, which are shown in figures 2E and F. To gauge the variation in the standardized spectra the error bars are shown that represent the standard deviation in the spectra at each frequency point. The inset in figure 2E shows an expanded view of the averaged spectra in the 1340-1360 cm\(^{-1}\) region. The error bars are more easily visualized in the inset. Importantly, there is no overlap of the error bars in the 1340-1360 cm\(^{-1}\) region, likely indicating a real difference between the spectra of mock and HSV-1 infected samples.

Statistical Analysis: The question we wish to address in this manuscript is: To what extent can we specify whether a given spectrum is associated with a mock, HSV-1 or Ad-5 infected cells? That is, what are the specificities and sensitivities associated with our FTIR spectral detection methods. Sensitivity is the probability that we can correctly identify a spectrum from an infected sample in the population of all spectra measured for infected samples (i.e. a true positive). Specificity is the probability that we can identify a spectrum from an uninfected sample given the whole population of spectra from uninfected samples (i.e. a true negative).

For each of the 728 frequencies in the standardized spectra in figure 2C, D we applied the Wilcoxon Rank Sum Test (also known as the Mann-Whitney test) to establish which frequencies may be useful in discriminating HSV-1 and Ad-5 infected samples from uninfected (mock) samples. The Wilcoxon Rank Sum Test is a non-parametric test for assessing whether two sample sets of observations come from a distribution with the same ranks. The Wilcoxon Rank Sum Test differs from the two-sample T-test, which essentially tests for differences in the means of two
sample sets. The Wilcoxon Rank Sum Test is more robust against outliers than the two-sample T-test.

The null hypothesis is that there is no difference in distributions. A two-sided alternative is that there is a difference in distributions. For a normal approximation to the rank distribution we can assign a score (Z score) relating to the probability that the given frequency can or cannot discriminate between HSV-1 and mock infected cells, or Ad-5 and mock infected cells. The calculated Z-score at each frequency for mock and HSV-1, mock and Ad-5, HSV-1 and Ad-5, and mock and (HSV-1+Ad-5) are shown in figure 3A-D. A second validating data set obtained from a completely different sample preparation was also analyzed. The corresponding calculated Z-scores for these validating data sets are also shown in figure 3 (red curves).

We calculate that for a Z-score of +/-4 the data can be used as a potential discriminator at the p=0.05 level: Using Bonferroni's multiple test method, we divide the significance level by the number of tests [0.05/(2*728)=3.434x10^-5] and then recognize that the percentile for a standard normal distribution with probability 3.434x10^-5 is -3.98 (~-4). In figure 3A-D the Z=+/-4 lines are shown. All points above +4 and below -4 may be useful for discrimination.

Mock vs. HSV-1 infected cells: For the original data for mock vs. HSV-1 (blue curve in figure 3A), there are seven broad frequency ranges that are all above the +/-4 threshold [800-885, 918-1014, 1036-1136, 1160-1207, 1216-1288, 1312-1388 and 1410-1500 cm^-1], and we focus only on frequencies in these ranges. For the Z-score data in figure 3A, 595 frequencies are above the +/-4 threshold, and are deemed significant. To generate a more stable methodology only the average of every 5 neighboring frequencies were considered. This leads to 119 significant frequency variables. Using stepwise selection in a logistic regression method frequencies at 1196, 1450 and 1484 cm^-1 were found to be of very high significance in discriminating spectra of mock
or HSV-1 infected samples. These three frequencies are indicated in figure 3A. These three frequencies all have a p-value less than 0.002. It is not at all obvious that these frequencies are the most significant from looking at the spectra in figure 2E.

To evaluate the performance of our logistic regression model in correctly classifying the two categories (mock vs. HSV-1) we calculated the sensitivity, specificity, and the area under the Receiver Operating Characteristic (ROC) curve. Sensitivity gives a measure of the proportion of correct identifications among actual positives, while specificity gives a measure of the proportion of correct identifications among all negatives. A ROC curve is a plot of sensitivity versus (1-specificity). An area under the ROC curve (AUC) of 1 represents 100% accuracy of discrimination, and an area of 0.5 represents no accuracy. A rough classification guide is: AUC = 0.9-1.0 - excellent discrimination; AUC = 0.8-0.9 - good discrimination; AUC = 0.7-0.8 - fair discrimination; AUC = 0.6-0.7 - poor discrimination; AUC = 0.5-0.6 - no discrimination.

Table 1 lists the specificities and AUC’s obtained from the final linear regression model for HSV-1 and mock, for sensitivities of 95%, 90%, and 80%. For the spectra of mock and HSV-1 infected samples (figure 2C), with the calculated frequency variables of highest significance, we find that the AUC is equal to 0.97. We also find that for a given sensitivity of 95/90/80% a specificity of 0.899/0.957/1.00 is obtained, respectively (table 2). This is an outstanding level of discrimination.

Frequently regression model fitting procedures over-estimate the accuracy of the method. The regression model works well for one data set but less well for another. That is, the determination capability of the model shrinks. Procedures are available, however, for estimating to what extent the model may overestimate the true AUC, sensitivity, and specificity. Taking this
over estimation into account is what is usually referred to as shrinkage. Here we have used 3-fold balanced cross-validation to assess shrinkage in our logistic regression model.

In balanced three-fold cross-validation the original data is randomly split into three approximately equal subsets. Two subsets are used to construct the statistical model, and the third subset is used for validation. From this analysis, specificities, sensitivities and AUC’s are calculated for the training data as well as for the same model applied to the validating data. Shrinkage is calculated by subtracting the calculated specificities and AUC’s for the validating data from the training data. We repeated this process 100 times for different randomly prepared subsets and the average value of shrinkage obtained is subtracted from the results calculated using the complete data set.

After estimating shrinkage using cross-validation methods, the AUC is equal to 0.963 (table 1). In addition, for a sensitivity of 95/90/80% we find a specificity of 0.82/0.938/0.989 (table 1). This still represents excellent discrimination of spectra for mock and HSV-1 infected cells.

To further validate our logistic regression model we repeated the experiments described above for a completely new set of mock, HSV-1 and Ad-5 infected cells. In this case 81/79/84 spectra for mock/HSV-1/Ad-5 infected samples were collected, respectively. In the process of validation, we applied the new data to the final model constructed from the original data. We obtained the AUC and specificities without changing any of the coefficients or variables calculated in the original model. The AUC and specificities calculated using the validating data are also listed in table 1. The Z-scores calculated for the new validating data are also presented in figure 3 (red curves).
Using the validating data in the logistic regression model yields an AUC of 0.935 (table 1). The calculated specificities are 0.95 for all three sensitivities. Such high scores may be expected given the similarity of the two Z-score curves in figure 2A. In particular, the discriminator frequencies calculated from logistic regression for the first data set, are also significant in the validating data set.

In an alternative statistical analysis of the spectra for mock and HSV-1 infected samples we used all 119 frequency variables (the 595 most significant ones from the Z-score plot averaged into groups of 5) to construct a partial least squares regression (PLSR)17, 18 model. In the PLSR model constructed using the data in figure 2C, it was found that the first 5 factors account for ~93.4% of the total variation (table 10, and contain almost all of the information from the original 119 variables. The AUC and specificities corresponding to 95%, 90%, and 80% sensitivity are also listed in table 1, and are all found to be equal to 1.0, demonstrating that our PLSR model is superior to our already excellent logistic regression model. The AUC and specificities obtained after estimating for shrinkage using 3-fold cross-validation are equal to 0.983, 0.999, 1 and 1 (table 1). Finally after using the new data to validate the constructed model, the AUC and the specificities are 0.989, 0.974, 0.975 and 0.988 respectively (table 1).

Mock vs. Ad-5: Figure 2D and F compares the standardized spectra and averaged spectra for mock and Ad-5 infected cells, respectively. The calculated Z-score plot for mock vs. Ad-5 is shown in figure 3B. For the red curves (original data sets) in figure 3B, the 925-953, 1021-1136, 1173-1206, 1219-1271, 1311-1392, and 1410-1500 cm⁻¹ regions are deemed significant. These regions contain 420 frequency variables that are reduced (stabilized) to 84 by averaging every five neighboring variables.
For spectra of mock and Ad-5 infected cells stepwise selection indicated that frequencies of 1057, 1134, 1470 and 1499 cm\(^{-1}\) are the most significant discriminators. These frequencies are labeled in figure 3B. Results for the logistic regression model using these four frequency variables are listed in table 2. As shown in table 2, using logistic regression, the AUC and specificities for 95\%, 90\%, and 80\% sensitivities are all unity, exhibiting excellent discrimination between spectra of mock and Ad-5 infected cells. After estimating shrinkage using cross-validation, and also using new validating data in the original model, the AUC and specificities are still very close to 1.0. The Z-score plot for both of the original and validating data are shown in figure 3B. There are some difference between the two Z-score curves, and one of the frequency variables is near the +/-4 borderline. This does not appear to lead to poorer discrimination, however.

For PLSR of the mock vs. Ad-5 spectral data, the first 4 factors account for \(~95.8\%\) of the total spectral variation. The AUC and specificities corresponding to 95\%, 90\%, and 80\% sensitivities are equal to 0.982, 0.958, 0.986, and 1. Again, such numbers indicate excellent discrimination, which is not greatly altered after estimating for shrinkage using cross-validation, or using new spectral data files for validation.

HSV-1 vs. Ad-5 infected cells: The calculated Z-score plot for HSV-1 vs. Ad-5 is shown in figure 3C. For the initial data, the 800-881, 915-938, 950-1026, 1146-1169, 1216-1297, 1336-1378, and 1413-1455 cm\(^{-1}\) regions are deemed significant. These regions contain 390 frequency variables, which is reduced (stabilized) to 78 by averaging every five neighboring variables.

For spectra of HSV-1 and Ad-5 infected samples stepwise selection indicated that frequencies of 854, 922, 952 and 1276 cm\(^{-1}\) are the most significant discriminators. Results for the logistic regression model using these four frequency variables are listed in table 3. The AUC and
specificities for 95%, 90%, and 80% sensitivities obtained using logistic and PLS regression models are all close to 1.0, indicating excellent discrimination. Even after estimating shrinkage using cross-validation, the AUC and specificities are still close to 1.0. Applying the calculated models to new validating data, the AUC is now calculated to be 0.882 (logistic regression) or 0.913 (PLRS), which still represents excellent discrimination. However, specificities for 95%, 90% and 80% sensitivity are 0.646, 0.759, and 0.848, respectively. It appears that discriminating spectra for Ad-5 and HSV-1 infected samples is straightforward for cellular suspensions made at the same time and under identical conditions. It is however, more difficult to apply models constructed using one set of data to new data sets obtained from different samples prepared at different times. The Z-score curves calculated from the spectra for the HSV-1 and Ad-5 infected cells, for the original and the validating data sets are shown in figure 3C. There are some considerable differences between the two Z-score curves. The 951 and 921 cm$^{-1}$ frequency variables chosen from the first logistic regression model are near the border of being significant in the second validating data set. The other two frequency variables chosen from the first logistic regression model are both also highly significant in the second validating data set. The PLSR model also performs quite poorly for the two different spectral data sets for HSV-1 and Ad-5 infected cells, and for an 80% sensitivity a specificity of 0.911 is calculated. This is very good level of discrimination, although inferior to that discussed above for mock vs. HSV-1 and mock vs. Ad-5.

Mock vs. HSV-1 & Ad-5 infected cells: In a final analysis we decided to test how well our statistical modeling approach could discriminate spectra for infected and uninfected (mock) cellular suspensions. In this case all of the spectra for HSV-1 and Ad-5 infected suspensions are grouped into a single category labeled “infected”. The calculated Z-score curve for mock vs. HSV-1 & Ad-5 is shown in figure 2D. For the initial data, figure 2D reveals that the 800-885,
921-959, 973-1006, 1027-1137, 1165-1207, 1217-1279, 1310-1391 and 1410-1500 cm$^{-1}$ regions are significant. These regions contain 570 frequency variables, which is reduced (stabilized) to 114 by averaging every five neighboring variables. After stepwise selection, frequencies of 1053, 1219, 1422, 1446, and 1460 cm$^{-1}$ are found to be the most significant discriminators. Results for the logistic regression model using these five frequency variables are listed in table 4.

The AUC and specificities for 95%, 90%, and 80% sensitivities obtained using linear regression and PLSR models are all close to 1.0 (table 4), again indicating excellent discrimination capability between spectra from uninfected and infected cellular samples. Applying the calculated models to new validating data, the AUC is now calculated to be 0.689 (logistic regression) or 0.986 (PLSR). It appears that the linear regression model fails but the partial least squares regression model excels. Similarly, for the logistic regression model, for 95%, 90% and 80% sensitivities, specificities of 0.208, 0.375, 0.465, respectively are calculated, representing poor discrimination. For the PLSR model, however, specificities of 0.938, 0.975 and 0.975 are calculated, representing excellent discrimination. Clearly the PLSR model is more appropriate when one is interested in merely differentiating infected from uninfected cellular samples. This result is likely because the PLSR method considers linear combinations of all of the variables while the logistic regression method considers only certain variables that may be appropriate for one type of infection but less so for another type.

Discussion

Above we have used advanced statistical methods to demonstrate outstanding discrimination of spectra obtained for mock and viral infected cellular suspensions. These methods are poorly understood by most non-statisticians, and it is worth considering whether there are visually identifiable features in the spectra that can be used for discrimination.
Although the error bars on the two spectra in figure 2E or F overlap at nearly all frequencies, the averaged spectra for mock and HSV-1 infected samples (figure 2E) appear readily distinguishable in several frequency regions: 1/ Spectra for mock infected cells display higher absorbance in the ~892-800 cm\(^{-1}\) region than spectra for HSV-1 infected cells. 2/ Between ~1141-1022 cm\(^{-1}\) mock infected samples display higher absorption than HSV-1 infected samples. 3/ Between ~1211-1302 cm\(^{-1}\) mock infected samples display higher absorption than HSV-1 infected samples. 4/ Between ~1302-1386 cm\(^{-1}\) mock infected samples display lower absorption than HSV-1 infected samples. 5/ Between ~1409-1500 cm\(^{-1}\) mock infected samples display lower absorption than HSV-1 infected samples. Such observations are consistently made over several experiments (not shown). Similar, but less pronounced observations can be made upon comparing the averaged spectra for mock and Ad-5 infected samples (figure 2F).

Comparison With Previous Studies For the mock infected cell spectra in figure 2E (and 2F), for all 728 frequency points, the mean standard deviation (average error bar length) is 0.08341 while the minimum/maximum standard deviation is 0.02027/0.17625, at 905/1090 cm\(^{-1}\), respectively. In contrast, previous studies on Vero cell suspensions have estimated a standard deviation of +/-0.001 \(^{7,8}\). We have not been able to generate such highly reproducible spectra using the data collection approach outlined here.

Salman et al., (2002)\(^{5}\) studied Vero cells infected with HSV-1 at an MOI=0.5, at one or three days post infection. Salman et al., (2002)\(^{5}\) used trypsin to dissociate the cellular monolayers from the growth medium. We have found that there is even greater variation in the averaged spectra for samples prepared using trypsin treatment. We have therefore avoided such a procedure. We prefer to scrape cells from the surface of the growth medium.
Salman et al., (2002)\textsuperscript{5} observe a very large change in a band near 1080 cm\textsuperscript{-1}, which they associate with cellular phosphates. We believe that it is more likely that the changes attributed to viral infection by Salman et al., (2002) are associated with different scattering effects associated with uninfected and infected material, and possibly due to preprocessing induced artifacts associated with baseline correction and normalizing spectra to the amide I absorption band. This latter point is particularly important, as it has been shown recently that the amide I peak position, width and intensity is particularly sensitive to dispersion type artifacts\textsuperscript{2,19}.

Erukhimovitch et al., (2004)\textsuperscript{7} also used a trypsin treatment to prepare Vero cell suspensions infected with HSV-1. These authors compared uninfected Vero cells with cells that had been infected with HSV-1 at an MOI=1.0 at 48 hpi. Erukhimovitch et al., (2004) indicate that infected cells have a much higher absorption in the 1200-1400 cm\textsuperscript{-1} region compared to control cells (see also Huleihel et al.\textsuperscript{6}). This result is not in keeping with our data, which show that the HSV-1 infected samples display decreased absorption in the 1211-1302 cm\textsuperscript{-1} region compared to control samples. Erukhimovitch et al., (2004) also observe a considerable infection induced decrease in absorption near 1023 cm\textsuperscript{-1}. This result is also not in keeping with our data, which shows an isobestic point near 1023 cm\textsuperscript{-1}. This is important since these authors use the change in absorption at 1023 cm\textsuperscript{-1} to probe HSV-1 infection development as well as to monitor how polysaccharides effect infection development\textsuperscript{6}.

Finally, Erukhimovitch et al., (2004) indicate that a band at 862 cm\textsuperscript{-1} in control cells shifts gradually to 854 cm\textsuperscript{-1} upon infection with HSV-1. However, we do not observe a band at 862 cm\textsuperscript{-1} in spectra of uninfected Vero cells. We do observe a band at 853 cm\textsuperscript{-1} (fig. 2D and F). However, this band does not shift in spectra obtained for HSV-1 infected cells.
In summary, it appears that nearly all of the observations attributed to HSV-1 infection by Erukhimovitch et al., (2004) disagree with our data. Too little data is presented in Erukhimovitch et al., (2004) to speculate further on the origin of the discrepancies.

Conclusions

Infrared spectra obtained for Vero cells that are uninfected or infected with HSV-1 or Ad-5 appear to be distinguishable even by eye (for MOI=10 and 24 hours post exposure). Using advanced statistical methods to analyze the spectra confirms this point in a quantitative fashion. Only two data sets are considered here for mock, HSV-1 and Ad-5 infected cells, this analysis does therefore not allow for a rigorous investigation of sample to sample variation. This aspect will be explored in a future publication.

Acknowledgements

This work was supported by the Department of the Army (U.S. Army Medical Research Acquisition Activity, Contract grant number: W81XWH-06-1-0795, and the Georgia Research Alliance. The opinions or assertions herein are the private views of the authors and are not to be construed as reflecting the views of the Department of the Army or the Department of Defense. We acknowledge Drs. Pinhas Fuchs and Chadi Filfili for critically reviewing this manuscript.
References
TABLE LEGENDS

Table 1: AUC and specificities corresponding to sensitivities of 95%, 90% and 80% for mock vs. HSV-1 infected cellular samples.

Table 2: AUC and Specificities for 95%, 90% and 80% sensitivities for Mock vs. Ad-5 spectra.

Table 3: AUC and Specificities for 95%, 90% and 80% sensitivity for HSV-1 vs. Ad-5.

Table 4: AUC and Specificities for 95%, 90% and 80% sensitivities, obtained for spectra of Mock vs. HSV-1 and Ad-5.

Table 1

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<thead>
<tr>
<th>Logistic regression</th>
<th>Mock vs. HSV-1</th>
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<td>Initial Data (figure 2C, E)</td>
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<td>Area under the curve (AUC)</td>
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<td>Specificity for 80% Sensitivity</td>
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Partial Least Squares regression
Number of Factors = 5. Percent Variation Accounted for by 5 Factors = 93.4

| | | |
| Area under the curve (AUC) | 0.992 | 0.974 | 0.993 |
| Specificity for 95% Sensitivity | 1.000 | 0.951 | 0.965 |
| Specificity for 90% Sensitivity | 1.000 | 0.991 | 0.980 |
| Specificity for 80% Sensitivity | 1.000 | 0.996 | 1.000 |

Table 2

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<td>Specificity for 95% Sensitivity</td>
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<tr>
<td>Specificity for 90% Sensitivity</td>
<td>1.000</td>
</tr>
<tr>
<td>Specificity for 80% Sensitivity</td>
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</tr>
</tbody>
</table>

Partial Least Squares regression
Number of Factors = 4. Percent Variation Accounted for by 4 Factors = 95.8

| | | |
| Area under the curve (AUC) | 0.982 | 0.955 | 0.994 |
| Specificity for 95% Sensitivity | 0.958 | 0.853 | 0.965 |
| Specificity for 90% Sensitivity | 0.986 | 0.939 | 0.993 |
| Specificity for 80% Sensitivity | 1.000 | 0.971 | 1.000 |
### Table 3

**HSV-1 vs. Ad-5**

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**Partial Least Squares regression**

Number of Factors = 6. Percent Variation Accounted for by 4 Factors = 94.9

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### Table 4

**Mock vs. HSV-1 & Ad-5**

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**Partial Least Squares regression**

Number of Factors = 5. Percent Variation Accounted for by 4 Factors = 94.1

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<td>Specificity for 80% Sensitivity</td>
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FIGURE LEGENDS

Figure 1: Transmission visible images collected at 2 locations for uninfected (mock) Vero cell suspensions on zinc selenide windows (the yellowish hue in the images is caused by the zinc selenide windows). The images show regions near the edge of the sample suspension. IR light impinges on the sample through only the central square region, which is ~100 μm on one side. The suspensions were prepared as described in the methods section, and air dried prior to measurement. Images were subjected to contrast enhancing routines available in Adobe Photoshop 8.0.

Figure 2: (A) IR absorption spectra in the 1500-800 cm⁻¹ region, for (A) mock (black), and HSV-1 (red) infected samples and (B) for mock (black), and Ad-5 (red) infected samples. The mock infected cell spectra are the same in both A and B. All samples were obtained from a single preparation run. For HSV-1 and Ad-5 infected samples the MOI is 10 and data were collected at 24 hours post exposure. 69/79/93 spectra were collected for mock/HSV-1/Ad-5 infected samples. (C, D) The same spectra as in (A, B) after standardization. For standardization, the mean absorbance is subtracted from the spectrum, which is then divided by the standard deviation. Standardization is equivalent to vector normalization except for a scale factor. (E, F) (Thick lines) Average of the spectra in (C, D). The error bars represent the standard deviation (plus/minus) of all of the spectra shown in (C, D) at each frequency. The inset in E shows an expanded view of the 1360-1340 cm⁻¹ region, where the error bars are more easily visualized.

Figure 3: Z-statistic obtained from the Wilcoxon Rank Sum Tests plotted as a function of wave-number for (A) mock vs. HSV-1, (B) mock vs. Ad-5, (C) HSV-1 vs. Ad-5 and (D) mock vs. HSV-1 & Ad-5. The blue curves are for the original data while the red curves are for the validating data collected at a later date. The frequencies that are predicted to be of highest significance are explicitly indicated.

FIGURES
FIGURE 2A and B
FIGURE 2C and D
FIGURE 2E and F
FIGURE 3
2.2 Supplementary Information

2.2.1 Hierarchical Cluster Analysis (HCA)

In addition of the logistic regression and partial least square regression, we also employed Hierarchical Cluster Analysis (HCA) to the same data sets described previously. The description of this method can be found in chapter one. All data processing and image assembly were performed by the program CytoSpec [29]. This program differs from software products available from instrument manufacturers in that it was designed and written to operate on entire spectral (imaging) data sets, rather than individual spectra[41].

We compared spectra between mock infected and virus infected (HSV1 and Adv-5) samples using CytoSpec. Additionally, we constructed comparisons between the raw data and normalized data (vector normalized). The raw and vector normalized data, consisting of 64 spectra of uninfected samples (mock), 65 spectra of samples infected with HSV1 or 81 spectra of samples infected with Adv-5, were loaded into CytoSpec in the wavenumbers range of 1500–800 cm\(^{-1}\).

All spectra were processed using Hierarchical cluster analysis (HCA). First, an initial inter-spectral distance matrix was calculated using a D-Values algorithm (Equation 2.2.1.1). These distance matrices were then clustered into two groups using Ward’s Algorithm[42].

\[
d_{y1y2} = \frac{1}{n_i + n_j + n_k} \left[ (n_j + n_l) d_{jl} + (n_k + n_l) d_{kl} - n_l d_{jk} \right]
\]  

(2.2.1.1)

We also employed the technique of HCA imaging, as illustrated in Figure 2-1. The dendrogram representing different clusters can be seen on the left. On the top-right side is the chemical map corresponding to the spectral intensity. On the bottom-right is a final HCA map produced by cluster analysis.
2.2.2 Results

The results of the HCA cluster imaging approach are illustrated in Figure 2-2 below. Picture A: IR images of a two-class classification trial, using the raw data of Mock (first 64 spectra) and samples infected with HSV1 (last 65 spectra). Picture B: IR images of a two-class classification trial, using the raw data of Mock (first 64 spectra) and samples infected with Adv-5 (last 81 spectra). Picture C: IR images of a two-class classification trial, using the vector normalized data of Mock (first 64 spectra) and samples infected with HSV1 (last 65 spectra). Picture D: IR images of a two-class classification trial, using the vector normalized data of Mock (first 64 spectra) and samples infected with Adv-5 (last 81 spectra). Predicted Mock is red and infected
samples are in blue (HSV1 and Adv-5). If our HCA algorithm were to have worked with a 100% specificity and sensitivity, the image of Figure 2-2A should have the first 64 blocks in red (representing the first 64 Mock spectra input) and followed with 65 HSV1 all in blue. However, we have red and blue blocks intermingled, which indicates that our specificity and sensitivity are not 100%. Based on Figure 2-2 and Equations 1.3.5.6 and 1.3.5.7, we can calculate the specificity, sensitivity and area under the curve for this dataset. Results are shown in Table 2-1.

As mentioned previously, an AUC between 0.80 and 0.90 represents good discrimination; AUC between 0.70 and 0.80 represents fair discrimination; AUC between 0.60 and 0.70 represents poor discrimination; and AUC between 0.50 and 0.60 represents no discrimination. From the results shown in Table 2-1, we have good discrimination between Mock and HSV1 for the raw data set, and fair discrimination for all the rest. From the results of AUC, it is hard to claim that vector normalization can improve the results of the data analysis.
**Figure 2-2** IR imaging of samples by HCA clustering

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<th>Vector normalized data</th>
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3 STUDIES OF VIRAL INFECTED CELLS AT 8 12 AND 24 HPE

3.1 FTIR Micro-spectroscopy for Studies of Viral Infected Cells

Abstract

Fourier transform infrared (FTIR) microscopy is a promising analytical tool for detecting changes associated with viral infection in cells.

In this study, we used FTIR microscopy to study Vero cells infected with herpes simplex type 1 (HSV1), which afflicts both humans and animals. We performed 11, 10 and 18 independent experiments (trails) at 8, 12 and 24 hours post exposure (hpe), respectively, with spectra of mock infected (uninfected) and HSV1 infected cells. Spectral bands show consistent differences within trials between spectra of mock infected and HSV1 infected cells and between HSV1 infected cells at 8, 12 and 24 hpe.

We found absorbance intensity differences between mock and HSV1 spectra within trials at 1244 cm\(^{-1}\), 1450 cm\(^{-1}\) and 1424 cm\(^{-1}\). The best region for monitoring the progression of HSV1 infection in Vero cells is in the ~1100-1000 cm\(^{-1}\) spectral region. The most notable feature is that the “HSV1 minus Mock” FTIR difference spectra change sign for samples between 12 and 24 hpe. From the difference spectra alone, it is possible to distinguish between spectra from HSV1 infected and mock-infected samples within trials at 8, 12 and 24 hpe.

Furthermore, FTIR spectroscopy in combination with multivariate statistical analysis provides a very important insight into HSV1 infected and mock-infected sample identification. The high specificities under different sensitivities, very low shrinkage and high AUC all indicate that we can discriminate between spectra from HSV1 infected and mock-infected samples.
Introduction

Infrared (IR) micro-spectroscopy of cells in culture is a rapidly growing area of biological/medical research, the goal of which is to spectrally distinguish between cells in different physiological states [16, 43]. One hypothesis that has gained traction recently is that IR microspectroscopy can rapidly and accurately differentiate between uninfected cells and cells that have been infected with viruses. In this sense the cell is a sentinel or biosensor that signals the presence of the virus or pathogen. One crucial feature of the cell as a biosensor is that it has its own built-in signal amplifiers, which are the phosphorylation kinase cascades that occur upon infection. This gives rise to opportunities then for pathogen discrimination.

Rapid and accurate detection of viral infections is of importance not only in the realms of disease detection in humans, animals and plants, but also in the realm of homeland security, where a rapid detection and diagnosis of airborne and environmental biological agents is desirable.

Here we focus on viruses that infect humans, and use herpes simplex virus (type 1) (HSV1) as our model system. Our biosensor is the African green monkey kidney cell (Vero cell). So, Vero cells act as sensors of HSV1 infection, responding to the virus with morphological or biochemical changes that we detect spectroscopically.

HSV1 is predominantly involved with infections of the skin and central nervous system, and is difficult to distinguish from other possible infections caused by bacteria or fungi, particularly at the early stages of the infection. Isolation of the virus in cell culture and immunoassays are the main methods for the detection of herpes virus infections. Such methods are time-consuming and relatively expensive, and an alternative faster approach is desirable.
Recently, we have begun exploring the application of FTIR spectroscopy for detection of HSV1 [44], building on the work of others [5, 6, 15]. Previously, we showed that spectra collected from different spots on dried Vero cell suspensions were highly variable. This was in agreement with other work that showed that even the spectra from single cells could vary greatly [45]. The question we addressed was: given this spectral variability, could we still discriminate between infected and uninfected cells? In addition, we also addressed the question of how sensitive and how specific our approach was. We used logistic and partial least squares multivariate regression methods and found that for Vero cells infected with HSV1 at an multiplicity of infection (MOI) of 10 at 24 hours after exposure, we could discriminate uninfected and HSV1 infected cell suspensions with very high levels of sensitivity and specificity. We also found that we could apply statistical models obtained using one data set to other data sets and still achieve high levels of discrimination between uninfected and infected cellular suspensions. Thus two data sets were used, one to first build a statistical model and then a second to validate the constructed model.

Since this previous work we have repeated the above described experiments, up to as many as 17 times, and it appears that there is or could be a greater degree of inter-experimental spectral variability that could not be adequately assessed using only two data sets. In this manuscript we consider the process of spectral discrimination of infected and uninfected cells with due consideration given to the presence of both inter and intra-experimental spectral variability. This led us to repeat each experiment as many as 17 times. From these measurements we are able to determine if discrimination is feasible in the face of both inter and intra-experimental spectral variability.
Previously we used advanced multivariate regression methods to analyze the spectral data. Given the very high levels of sensitivity and specificity achieved we decided to test alternative, simpler statistical methods of data analysis in order to compare the various approaches.

In previous measurements we considered data collected at 24 hours post exposure. In this paper we consider the possibility of more rapid detection of HSV1 infections in cells and we consider spectra obtained for cells at 8 and 12 hours post exposure. In addition the question of whether the spectral changes are the same or display a temporal profile is addressed.

**Materials and Methods**

Vero cells (American Type Culture Collection #CCL-81) were grown as described previously\[44\]. For HSV1 infections cells were incubated in infected cell lysate at an MOI of 10 for one hour. Cells were then washed and reincubated for a further 8, 12 or 24 hours in virus free RPMI medium, as described previously \[44\].

Tyrosine and threonine phosphorylation of several proteins were known to be induced at 4 and 6 hours post exposure (hpe) in infected cells.

After incubation for 8, 12 or 24 hours, the cells were washed in PBS containing calcium and magnesium and scraped into the same buffer, as described. The cells were centrifuged at 800 g and resuspended in 5 μl of the fresh PBS. Samples were pipetted onto ZnSe windows, and air dried.

FTIR spectral data collection was identical to that described previously \[44\]. Data was collected in the 2000-800 cm\(^{-1}\) region but was truncated after collection to consider only the 1500-800 cm\(^{-1}\) region. Spectral data was collected at 2 cm\(^{-1}\) resolution. For any particular experiment spectra collected were vector normalized and then averaged.
Results

We have collected spectra for mock and HSV1 infected cellular suspensions at 24, 12 and 8 hours post exposure. Transmission visible images of the samples were similar to those presented previously [44]. Figures 1A, 1B and 1C show vector normalized FTIR spectra for mock and HSV1 infected samples at 24 (A), 12 (B) and 8 (C) hours post exposure (hpe), respectively. Spectra were collected for 11, 10 and 18 independently prepared mock and HSV1 infected samples, respectively. The spectra have been shifted vertically for comparison. Figures 1C, 1D and 1F show the same spectra as in 1A, 1B and 1C without the vertical shifting. Both views are required for an evaluation of the inter-experimental spectral variability. Certain spectral trends are observed in nearly all experiments on cells at 8, 12 or 24 hpe. These spectral trends differ for the three time points, however (see below).

To better visualize the similarities and the differences in the spectra in Figure 1, Figure 2 shows averaged spectra. If HSV1 and mock can be differentiated from the dataset, there will be a difference between the mean of the spectra density of HSV1 and mock. Therefore, Figure 2A compares average spectra for mock infected cells at 8, 12 and 24 hpe. The most noticeable spectral differences are around 1243 cm\(^{-1}\), 1083 cm\(^{-1}\) and below ~854 cm\(^{-1}\). In addition, the peaks of the 1243, 1342, 1455 and 1467 cm\(^{-1}\) bands are slightly decreased in the spectrum from mock cells at 8 hpe compared to the bands in the spectrum of mock cells at 12 and 24 hpe.

Figure 2B compares the averaged spectra for mock cells at 24 hpe with the spectra for HSV1 infected samples at 8, 12 and 24 hpe. The spectra for HSV1 infected cells clearly differ at 8, 12 and 24 hpe. This is most clearly observed for the peak near 1083 cm\(^{-1}\), which increases/decreases in intensity in the spectra of HSV1 infected cells at 8 and 12/24 hpe, respectively.
In the 1300-1500 cm\(^{-1}\) region, for the spectra obtained at 24 hpe, the spectra for HSV1 infected cells display increased absorption intensity compared to the spectra for mock samples. This behavior is observed in 16 of the 18 experiments (Fig. 1A). Such an observation is less clear-cut for the spectral data at 12 hpe. It is also not totally clear in the spectra in Figure 2B. To better visualize the spectral differences between the mock and HSV1 infected cells at the different time points Figures 3A, 3B and 3C show the “HSV1 minus mock” FTIR difference spectra for the 24, 12 and 8 hpe data, respectively. Figures 3A, 3B and 3C demonstrate considerable inter-experimental spectral variation. To more easily visualize the similarities and differences in the spectra at 8, 12 and 24 hpe, Figure 3D shows the average of the 8 hpe (green), 12 hpe (blue) and 24 hpe (black) “HSV1 minus mock” FTIR difference spectra. Figure 3D demonstrates differences in the spectra for HSV1 infected cells at 8, 12 and 24 hpe, particularly so in the 1169-989 cm\(^{-1}\) region, and below ~889 cm\(^{-1}\).

**Multivariate Statistical Analysis**

Visual inspection of the collected spectra (Fig.1 A-C) shows significant variation between measurements obtained on different days. This variation is considerably larger than expected from previous results from only two data sets[46]. The cause and impact of this issue is not yet fully understood, though it is apparent that we need to reevaluate our choice of statistical method(s). It should be noted that this variation is present despite our efforts to standardize the data collection procedure.

In order to eliminate the influence of this day-to-day variability, we must average all the spectra (~50-70) obtained in a day and treat the single averaged spectrum as an independent observation. By doing so, we reduce the sample size dramatically and also limit the statistical me-
methods we can choose. For example, the statistical methods which simply compare the means of two groups (mock vs. HSV1) will not work sufficiently with our data sets due to the small sample size.

Before applying the in-house multivariate statistical analysis, two steps must first be taken. These are the standardization and the smoothing of the spectra. Standardization entails subtracting the mean from an individual intensity and then dividing this difference by the standard deviation of the sample population. Smoothing of the spectra involves summing and averaging each four adjacent data points together. The spectral range is from $1500 \text{ cm}^{-1}$ to $800 \text{ cm}^{-1}$ and contains 728 data points. The smoothing procedure reduces these 728 variables by a factor of four into 182, which increases the reliability of the results of our statistical method.

After the standardization and smoothing, data in each day for each kind (mock or HSV1) were randomly split into two even groups and each group was then averaged into one spectrum. Two types of difference spectra can be calculated after this step. One type of difference spectrum, called **inner difference**, is obtained by taking the difference between two groups for a single kind within each day (Fig. 4: inner 1 and inner 2). The second type of difference spectrum, called **intra difference**, is obtained by subtracting the HSV1 spectra from mock for each group within each day (Fig. 4: intra 1 and intra 2). Thus, for each day of data at each time post exposure (8, 12 or 24 h) we produce two intra and two inner difference spectra. Note that the inner difference spectra would ideally be flat and featureless, while the intra spectra should not.

Statistical methods can then be performed on the inner and intra difference spectra. Since we are assuming the inner differences are zero, and since we are comparing intra spectra to the inner spectra, we are essentially seeing how the intra spectra differ from zero. Thus, a one sample test, the Wilcoxon Signed-Rank Test (WSRT), would be more appropriate than a two sample
test, the Wilcoxon Rank Sum Test (the Mann-Whitney Test) that we used previously [46] for picking the most significant wavenumbers.

Figure 7 shows the p-value results from the WSRT. The significant wavenumbers were determined according to the Bonferroni correction [47]. The criterion of p-value is equal to 5% divided by 182 (the number of variables), which is nearly 0.0002. Therefore, only wavenumbers with a p-value < 0.0002 were selected. This selection criterion applies to all the data - inner and intra difference. It should be noted that the same wavenumbers were not selected for in different datasets.

We then applied Partial Least Square Regression (PLSR) [48] only on the selected wavenumbers. According to central limit theory, since we averaged the datasets, we can calculate sensitivity, specificity and Area Under the Receiver Operating Characteristic (ROC) Curve (AUC) based on the predicted values of the results from PLSR for the observed datasets.

Since our data size is not large enough to represent the whole population, shrinkage [49] becomes an important indicator for the future performance of this model in discriminating the unknown future data. Shrinkage is defined as the difference between the specificities (or sensitivities) of two data sets: the population and the observed sample. In order to estimate the shrinkage, we calculated the difference between the specificities of the model data sets and test data sets.

We employed two methods to determine the model and test datasets. First, we applied 2-fold cross validation, which normally gives an under-estimated shrinkage. The intra or inner differences were split into model and test groups, called model intra and test intra or model inner and test inner. We applied WSRT only on the model intra data to determine the significant frequencies. PLSR was then applied to the selected frequencies for model intra and model inner da-
ta sets. By applying the same discriminators for PLSR as for the model intra and inner sets, we were able to calculate the predicted values for the test sets. We calculated sensitivity, specificity and AUC from the predicted values of PLSR for both model and test sets. By finding the difference between these two specificities, we obtained estimates of the shrinkage (Table 1, Column 3). Specificity of Cross Validation (Table 1, Column 4) was calculated by subtracting this shrinkage (Table 1, Column 3) from the specificity for original data (Table 1, Column 2).

The second method we used to determine the model and test datasets was the bootstrap method, which normally gives an over-estimated shrinkage. Model datasets for bootstrap were calculated by constructing a number of resamples. Resampling entails drawing randomly with replacement from a set of data points of the observed dataset, which are of equal size to the original observed datasets. Test datasets refers to the original observed dataset. By applying WSRT only on the model intra data, we determined the significant frequencies. PLSR was then applied to the selected frequencies for model intra and inner data. By applying the same discriminators for PLSR as for model datasets, predicted values of PLSR were calculated for the test datasets. Following the same procedures as for CV, we calculated column 5, shrinkage from Bootstrap, and Column 6, Specificity calculated using Bootstrap. Col 6 was obtained by subtracting column 5 from column 1. NOTE: format the ending sentences of the last two paragraphs the same way.

By finding the average of these two specificities, as in column 7, we can have fairly accurate final specificities and AUC for three different sensitivities. All multivariate analysis was performed using SAS 9.1 (SAS Institute Inc., Cary, NC, USA).
Discussion

A visual inspection of the spectra reveals some differences in the spectra of mock infected samples at 8, 12 and 24 hpe. This may not be expected. However, the Vero cells grow as monolayers, and 24 hpe is one full day of growth of cells once they have already formed a confluent monolayer. We have observed the very beginnings of cytopathic effects in the monolayers in visible images of mock samples at 24 hpe. It may therefore not be totally surprising that there are minor differences in the spectra in Figure 2A again showing that our data has potential variation between days. The difference between the spectra for mock samples in Figure 2A can be considered as a measure of the noise in the experiments (admittedly the spectrum for the mock sample at 24 hpe in Figure 2A is the average of nearly 700 spectra spread over 17 measurements). In addition, each of the nearly 700 spectra results from the co-addition or average of 64 interferograms.

The spectra in Figures 1D, 1E and 1F show that there is not a single frequency in the 800-1500 cm\(^{-1}\) region where all of the spectra for mock samples display a lower or higher absorbance than that found in all of the spectra for HSV1 samples. In fact, the inner-experimental spectral variability (mock in day 2 minus mock in day 1) is not considerably less than the intra-experimental spectral (HSV1 in day 1 minus mock in day 1) variability (compare Figure 1C in this manuscript with Figure 2C in our previous manuscript [44]). This is in spite of the fact that we have taken some care in manually choosing the area of the sample suspension from which spectra are measured! We have measured FTIR spectra for 17 independently prepared mock and HSV1 sample suspensions for 24 hpe. This is a very large set of data by most standards. The conclusion is that simple spectral comparisons will not greatly improve the sensitivity and
specificity with which we can distinguish spectra associated with mock and HSV1 infected samples.

There are some very clear trends in the spectra in Figure 1D (the trends are less clear in the spectra in Figures 1E or 1F). From Table 1, the application of advanced multivariate statistical methods have shown that very high levels of sensitivity and specificity (near equal to 100%) in discriminating spectra from mock or HSV1 infected samples at 8, 12 and 24 hpe (MOI=10) are possible when large numbers of frequencies are simultaneously considered [44], and we have an excellent discrimination between mock and HSV1 for all 24, 12 and 8 hpe. One conclusion that can be drawn is that even though we cannot monitor the large differences in the spectra between the mean of the infected and uninfected samples, by applying the right statistical analysis, and we can still discriminate the two samples successfully with a very high certainty.

Principle component fed liner discriminate analysis with leave-one-out cross validation (PCA with LOOCV) [50] is another method that has been applied to do the discriminating analysis between two or more groups. We believe this is an inappropriate method to use when the datasets consist of several measurements in different days. Even the improved result from this method does not indicate better discrimination. Since our data had a large variation day-to-day, spectra within each day were not treated as independent measurements. Therefore, cross validation with just leaving one single spectrum, but not the spectra from at least a whole day, out each time for LDA is no longer a fair validation method for our dataset. Furthermore, as we have shown, there exists significant variation between measurements obtained on different days. It is thus very important to measure samples on different days in order to properly represent the real population.
In all of the experiments undertaken, spectra were collected for both mock and HSV1 infected samples that were prepared at the same time. A question one could ask: Is it possible to use an arbitrary spectrum for mock samples (from Figure 1A or 1B or perhaps the averaged spectrum in Figure 2A) and use it as a standard for assessing if an unknown spectrum came from a mock or HSV1 infected sample? It is of course possible to follow this procedure. The real question is, however, what kind of specificity and sensitivity could be associated with following such a procedure? The question can also be considered as, can we build spectral libraries using the spectra in Figure 2A as standards? If we do this, what level of specificity and sensitivity could be associated with assigning an arbitrary spectrum from an unknown sample?

Another conclusion we may draw is that it is not possible to take a mock spectrum from any particular day’s measurements and use it as a standard for comparison to spectra for HSV1 infected samples on different days. The important conclusion is that spectra for mock and HSV1 infected samples need to be collected together for a proper analysis.

If spectra are always collected for mock and infected samples together then some clear conclusions can be derived from the “HSV1 minus mock” FTIR difference spectra (DS) in Figures 3A, 3B and 3C. Firstly, for cells at 24 hpe, the FTIR DS in all 18 experiments display a negative intensity at ~1056 and ~1227 cm\(^{-1}\), and a positive intensity at ~1450 and 1424 cm\(^{-1}\). These therefore appear to be very good frequencies for distinguishing spectra from mock versus HSV1 infected samples at 24 hpe, at an MOI of 10. The same kind of simple analysis can be applied to the FTIR DS in Figure 3B or 3C and different significant frequencies can be established.

From the FTIR DS in Figure 3D, if a positive signal is observed over the whole of the 1300-1500 cm\(^{-1}\) region then we are likely dealing with HSV1 infected cells that are near 24 hpe
(at MOI=10). Furthermore, as can be seen in Figure 3D, a trend of decreasing intensity of the difference spectra between HSV1 and mock can be detected as the time post-infection decreases from 24 to 8 hpe.

The FTIR DS in Figures 3A-3D indicate that by far the best region for monitoring the progression of HSV1 infection in Vero cells is in the ~1100-1000 cm\(^{-1}\) spectral region. The most startling feature is that the FTIR DS changes sign for cells at 8 and 12 or 24 hpe around 1100 cm\(^{-1}\) (see Figure 5), making this a very significant area.

It is clear from the spectra in Figure 2B that spectral changes caused by HSV1 infection in Vero cells are quite small. Only small intensity changes are observed, and no band frequency shifts are observed. This is in spite of the fact that the MOI is 10. For an MOI of say 1.0 we could suggest that the spectral changes caused by infection would probably be insignificant at 24 hpe, and could probably only be inferred by applying multivariate statistical methods to the analysis of the spectral data. Interestingly, however, Salman et al [51] have produced FTIR spectra for uninfected Vero cells and Vero cells infected with HSV1, at an MOI of 1.0, at 24 hpe. The spectral differences between mock and HSV1 infected cells at 24 hpe at an MOI=1.0 are at least as large as the changes observed for an MOI=10.

Erukhimovitch et al. 2004 [6] also obtained FTIR spectra for Vero cells infected with HSV1, at an MOI=1.0 at 24 hpe. After baseline correction of the spectra and then vector normalization, these authors found that HSV1 infection led to the loss of a band at 1023 cm\(^{-1}\) [5, 6]. The 1023 cm\(^{-1}\) band was assigned to carbohydrates, apparently because glycogen in water was shown (using a diamond ATR accessory) to display a band at 1025 cm\(^{-1}\) [52]. A band at 1023 cm\(^{-1}\) is not observed in our spectra for HSV1 infected Vero cells at 24 hpe, at an MOI=10.0.
**Figure 1:** FTIR spectra collected for mock (*black*) and HSV1 (*red*) infected cells at 24 (A), 12 (B) and 8 (C) hours post exposure, for 18 (A), 10 (B) or 11 (C) independent experi-
ments. Cells were infected with HSV1 at an MOI of 10 for one hour, and then washed. Cells were then incubated for a further 24/12 hours in virus free RPMI medium. Each spectrum shown is the average of 25-70 spectra collected for each sample suspension. The spectra were vector normalized but not baseline corrected. Spectra have been shifted vertically. (D), (E), (F) Same spectra as in (A), (B), (C) except the spectra have been shifted vertically.

**Figure 2:** (A) Average of the spectra for mock cells in Figure 1 at 8 (blue), 12 (red) and 24 (black) hpe. (B) Average of the spectra for mock cells in Figure 2A at 24 (black) hpe. Also shown are the averages of the spectra for HSV1 infected cells (from Figure 1) at 8 (green), 12 (blue) and 24 (black) hpe.
Figure 3: “Mock minus HSV1” FTIR difference spectra calculated for each of the experiments at 24 hpi (A), 12 hpi (B) and 8 hpi (C) shown in Figures 1A, 1B and 1C, respectively. (D) Averaged “mock minus HSV1” FTIR difference spectra of the 8 hpi (red), 12 hpi (blue) and 24 hpi (black) “HSV1 minus mock” FTIR difference spectra shown in Figure 2A, 2B and 2C, respectively.
Figure 4: Simple methodology for the data preprocess and multivariate statistical analysis.
Figure 5: “Inner verses Intra” FTIR difference spectra calculated for each of the experiments at 24 hpe (A), 12 hpe (B) and 8 hpe (C) shown in Figure 1A, 1B and 1C with 36 (A), 20 (B) or 22 (C) difference spectra (inner or intra), respectively. Inner is the difference spectra of 2 groups of mock and HSV1, separately within each day (blue); Intra is the difference spectra between HSV1 and mock for 2 groups within each day (red). We shifted each time point up for easy comparison. Around 1100 cm$^{-1}$, 24 hpe has a different trend as opposed to 12 and 8 hpe, which also matches with our visual spectra comparison. Around 1200-1300 cm$^{-1}$, all three time points have similar features for discriminating the intra from inner difference. This is also shown in Figure 5, from the overlay of all spectra from Figure 4.
**Figure 6**: Overlay of all spectra from Fig.4 with inner (*black*) and intra (*red*) for 24 hpe (Fig 5A), 12 hpe (Fig 5B) and 8 hpe (Fig 5C).

**Figure 7**: Plot for p-value results from WRST for all the wavenumbers between 1500 cm⁻¹ and 800 cm⁻¹ for 24 hpe (*black*), 12 hpe (*red*) and 8 hpe (*blue*). The black horizontal line equals 0.05.
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<td>0.999899</td>
<td>0.965416</td>
</tr>
<tr>
<td>AUC</td>
<td>1</td>
<td>0.070872</td>
<td>0.929128</td>
<td>0.002237</td>
<td>0.997763</td>
<td>0.963445</td>
</tr>
</tbody>
</table>

**Table 1**: Results from multivariate statistical analysis for 24 hpe (Table A), 12 hpe (Table B) and 8 hpe (Table C).
3.2 Supplementary Information

3.2.1 Principal Component Discriminant Analysis (PCA-LDA)

The raw .csv files were processed using the PCA-LDA method as well as the leave-one-out cross-validation method. All multivariate analysis was performed using SAS 9.1 (SAS Institute Inc., Cary, NC, USA).

To analyze the dataset, principal component analysis (PCA) was first applied to the data set to reduce its dimensionality by computing the selected eigenvectors of the covariance matrix. Next, PCA scores were used as an input for linear discriminant analysis (LDA) in which the class variables corresponded to the differentiation treatments for vero cells (Mock=0, HSV1=1, ADV=2). Again, LDA was employed to project the PCA scores to a new space to find the most discriminative directions, which allowed the largest difference to be shown.

As recommended in Babrah (2008), we also used first 25 PCs as input for LDA [30]. The LDA results showed that our dataset had a good discrimination between different infections (Table 3.9), which proved that 25 PCs is enough for the discriminant analysis for this dataset.

We also employed the same type of analysis for the T-cell data. It turned out that a much smaller number of PCs was needed, which I will expand upon in a later chapter. Therefore, how many PCs were chosen mostly depended on the dataset itself and required accuracies (Sensitivity, specificity or AUC).

The classification accuracies were computed by performing the leave-one-out cross-validation (LOOCV) method. LOOCV is the degenerate case of K-Fold Cross Validation, where K is chosen as the total number of spectra. Leave-one-out cross-validation involves using a single observation from the original sample as the validation data, and the remaining observations
as the training data. This is repeated such that each observation in the sample is used once as the validation data.

### 3.2.2 Results

The figures below are scatter plots for the first two LDA factors for 24h, 12h and 2h data. The horizontal axis is the first canonical component and vertical axis is the second canonical component produced from LDA. The plot of first two LDA factors is a good representation of the efficacy of using the PCA-LDA to show the separation between two or three groups of data.

For 24 hpe in Figure 3-1, the x-axis is the first canonical component and y-axis is the second canonical component produced from LDA. Figure A is a scatter plot between mock (black) and HSV1 (red) infected samples. Figure B is a scatter plot between mock (black) and Adeno (red) infected samples. Figure C is a scatter plot between HSV1 (black) and Adeno (red) infected samples. Figure D is a scatter plot of mock (black), HSV1 (red) and Adeno (green) infected samples. For 12 hpe in Figure 3-2, the horizontal axis is the first canonical component and vertical axis is the second canonical component produced from LDA. Figure A is a scatter plot between mock (black) and HSV1 (red) infected samples. Figure B is a scatter plot between mock (black) and Adeno (red) infected samples. Figure C is a scatter plot between HSV1 (black) and Adeno (red) infected samples. Figure D is a scatter plot among mock (black), HSV1 (red) and Adeno (green) infected samples. For 2 hpe in Figure 3-3, the x-axis is the first canonical component and y-axis is the second canonical component produced from LDA. Figure A is a scatter plot between mock (black) and HSV1 (red) infected samples. Figure B is a scatter plot between mock (black) and Adeno (red) infected samples. Figure C is a scatter plot between HSV1 (black) and Adeno (red) infected samples. Figure D is a scatter plot between mock (black), HSV1 (red) and Adeno (green) infected samples.
and Adeno (red) infected samples. Figure D is a scatter plot among mock (black), HSV1 (red) and Adeno (green) infected samples.

It is clearly indicated that there is a good separation between mock and HSV1 and also between HSV1 and Adeno infected samples (Fig.3-1 A and C), but for mock vs. Adeno, it is hard to distinguish them from each other (Fig.3-1 B and D). A similar trend can be observed in the sample of 12 hpe (Fig. 3-2), as well as the 2 hpe data. The overlapping in 2 hpe in Fig. 3-3 B is considerably more than in Fig. 3-3 A and C. The most significant feature is that as the infection time reduced, it became harder to distinguish between mock and Adeno infected samples, which agrees with the analysis results in previous paper [53].

In summary, it is possible to use canonical discriminant analysis to discriminate between these different infections by using two canonical components that are linear combinations of the PCs derived from spectral intensity. However, it should be noted that this analysis method cannot distinguish between all groups equally well. We speculate that there is not enough spectral difference between mock and Adeno infected samples for this method to be used effectively.
Figure 3-1 Plots of first 2 LDA factors for 24hpe
Figure 3-2 Plots of first 2 LDA factors for 12hpe.
Figure 3-3 Plots of first 2 LDA factors for 2hpe.
4 FTIR STUDIES OF VIRAL INFECTED CELLS AT 2 4 6 HPE

4.1 FTIR Micro-spectroscopy for Studies of Viral Infected Cells at Different Hours Post Exposure

Abstract

Fourier transform infrared (FTIR) microscopy is a promising analytical tool for detecting changes associated with viral infection in cells.

In this study, we used FTIR microscopy to study Vero cells infected with herpes simplex type 1 (HSV1). We performed a total of 63 independent experiments (trials) at 2, 4 and 6 hours post exposure (hpe) at 21 trials per time point, with spectra of mock infected (uninfected) and HSV1 infected cells. Spectral bands show consistent differences within trials between spectra of mock infected and HSV1 infected cells and between HSV1 infected cells at 2, 4 and 6 hpe.

There were absorbance intensity differences between mock and HSV1 spectra within trials at 1450-1300 cm\(^{-1}\), 1200-1000 cm\(^{-1}\), and 850-800 cm\(^{-1}\). The best region for monitoring the progression of HSV1 infection in Vero cells is in the ~1100-1000 cm\(^{-1}\) spectral region. The features that the “HSV1 minus Mock” FTIR difference spectra change sign for cells between 4 and 6 hpe is around 1460 cm\(^{-1}\), 1244 cm\(^{-1}\) and 920-860 cm\(^{-1}\). From the difference spectra alone, it is very possible to distinguish between spectra from HSV1 infected and mock-infected samples within trials at 2, 4, and 6 hpe.

In our continuing research, we have found that FT-IR spectroscopy in combination with multivariate statistical analysis provides very important insights into the identification of HSV1 infected and mock-infected samples. The high specificities under different sensitivities, very low
shrinkage and high AUC all indicate that we can discriminate between spectra from HSV1 infected and mock-infected samples.

**Introduction**

Infrared (IR) micro-spectroscopy of cells in culture is a rapidly growing area of biological/medical research, the goal of which is to spectrally distinguish between cells in different physiological states [16, 43]. As was mentioned in the last chapter, there were many notable features found in the spectra, which could help distinguish mock infected from herpes simplex virus (type 1) (HSV1) infected samples at 8, 12 and 24 hours post exposure (hpe), according to difference spectra analysis and statistical analysis. Since rapid and accurate detection of viral infections is very desirable, here we shorten the post exposure time and focus on Vero cells infected by HSV1 at 2, 4 and 6 hpe as our model system. Again, our biosensor is the Vero cell. Vero cells act as sensors of HSV1 infection, responding to the virus with morphological or biochemical changes that we detect spectroscopically.

In previous measurements we considered data collected at 8, 12 and 24 hours post exposure. In this paper we consider the possibility of more rapid detection of HSV1 infections in cells and we consider spectra obtained for cells at 2, 4 and 6 hours post exposure. In addition the question of whether the spectral changes are the same between time points, or display a temporal profile, is addressed.

Previously, we showed that spectra have consistent differences within trials between spectra of mock infected and HSV1 infected cells and between HSV1 infected cells at 8, 12 and 24 hpe around ~1100-1000 cm$^{-1}$, 1244 cm$^{-1}$, 1450 cm$^{-1}$ and 1424 cm$^{-1}$ region. We also noticed features that the “HSV1 minus Mock” FTIR difference spectra change sign for samples between
12 and 24 hpe, which can be used to distinguish between spectra from HSV1 infected and mock-infected samples within trials at 8, 12 and 24 hpe. We have repeated the above-described experiments, up to as many as 18 times, and it appears that there is an increased degree of spectral difference as the infection time decreasing from 24 to 8 hours. This led us to repeat each experiment as many as 21 times for shorter infection time points in order to observe the differences caused by infection.

**Materials and Methods**

**Vero Samples Preparation**

African green monkey kidney cells (Vero cells, American Type Culture Collection #CCL-81) are treated as sample cells, and infected by different kinds of viruses (herpes simplex type 1 (HSV1), adenovirus type 5 (Ad-5), coxsackie virus and measles virus), which infect both humans and animals. The samples were 1 or 2 µl drops of sucrose gradient-purified HSV-1 in a buffer containing 10 mM tris, 150 mM NaCl and 1 mM EDTA, grown at 37 °C. Vero cells were grown at 37°C in DMEM (Mediatech) supplemented with 10% Fetal Plus Serum (Valley Biomedical), 5 µg/ml penicillin, and 5 units/ml streptomycin (Mediatech). Cells were seeded on 13 mm diameter x 1 mm thick zinc selenide (ZnSe) windows (for transmission experiments) that had been sterilized in 95% ethanol and air dried. The windows were immersed in the growth medium in 6 or 12-well cell culture plates. Approximately 7x10e5 viral particles were present in each sample. For HSV-1 strain F (purified), it was the lysate stock infecting the cells at a multiplicity from 10 PFU per cell (MOI=10). Tyrosine and threonine phosphorylation of several proteins were induced at 4 and 6 hpi in cells infected with purified virions. In cells infected with lysate viral stock, phosphorylation of ~35 kDa protein were likely induced by the cellular factors from
the infected lysate stock. At different (2, 4 and 6) hours post infection (hpi), the cells were washed in PBS containing calcium and magnesium and scraped into the same buffer and no trypsin treatment was used for enzymatic disaggregation. The cells were centrifuged at 800 g and resuspended in 5 μl of fresh PBS. Samples were pipetted onto ZnSe windows, which are widely used due to their strong absorption in the IR wavenumber range, and cells were air dried. The cells were examined under a light microscope for the appearance of the cytopathic effect (CPE) after 2, 4 or 6 hpi. The CPE is defined as the percentage of damaged cells in the focusing area[53].

Here is the detailed procedure for sample cell preparation.

1- Seed 6-well plates overnight with ~ 700,000 cells/well in MEM supplemented with 10% FBS, 100 U/ml Pen-Strep and 2mM L-glutamine. The expected final count after O/N incubation is 1 x 10^6 cells per well.

2- Next day virus suspensions are prepared at an MOI of 10 using HSV-1 as an example:
   a. HSV-1 (F; AAJA) titer = 4 x 10^8 PFU/ml.
   b. Number of cells per well = 10^6 cells
   c. Total cells in 6 wells (one for each time point) = 6 x 10^6 cells
   d. For MOI=10; PFU needed = 6 x 10^7 PFU
   e. Total Volume of stock of HSV-1 = (6 x 10^7 PFU) / (4 x 10^8 PFU/ml) = 0.15 ml.
   f. 6 ml of 2% heat inactivated FBS are added to 150 ul of stock, mixed by brief vortexing; media in 6-well removed and replaced with 1 ml of virus suspension.

3- Note that for adenovirus the main stock adenovirus type 5 is 10^9 NAS units/ml. Experimentally it has been determined that the addition of 100 μl of this stock is able to completely infect 1 x 10^7 cells. Therefore, 100 μl of the adenovirus stock is estimated to be an
MOI of at least 10 for each well. So 600 µl of stock are added to 5.7 ml of media and used to infect 6 wells, one for each time point.

4- At 2 hours post infection, remove media containing virus from all wells and replace with 2 ml of 2% FBS supplemented MEM. Infected cells from the 2h time point plate are also collected per the steps below at this time.

5- Take pictures for CPE for documentation and remove media from wells of the 2 hour plate; replace with 1 ml of ice cold DPBS with Calcium and Magnesium per well. Using a cell scraper, scrape the monolayer into DPBS and transfer into 2 ml microcentrifuge tube with O-ring cap

6- Centrifuge for 2 minutes at 2000 g.

7- Wipe the ZnSe window with an isopropanol wipe if necessary and air dry on the biosafety cabinet grate. Divide the window into sections using a permanent marker pen.

8- Remove all DPBS from the tube using vacuum attached to a Pasteur pipette; vortex the cell pellet briefly.

9- Transfer 0.5 ul of the pellet and dot on the ZnSe window. Segregate dots into sections by virus type. Let the dots air dry on the biosafety cabinet grate.

10- Take pictures of one dot for each virus; place the window in a clean container (e.g. 35 mm culture dish), label with the time post infection, date and a scheme of the ZnSe window and dot distribution.

11- Place in secondary clean container and transfer to Physics lab for FTIR analysis.

12- Similar procedures were performed to cells with different MOI range from 0.31 to 10 (MOI=0.31-10) for statistical analysis and unknown test.
FT-IR Experiments and Instrumentations

FTIR microscopy measurements were undertaken using a Varian 7000 FTIR spectrometer (Varian, Lake Forest, CA) coupled to a Varian UMA600 IR microscope. The microscope was set up in transmittance mode. The microscope was equipped with a visible light source and CCD camera that allowed the collection of visible microscopic images from the same region that was sampled in the IR. The IR light was detected using a single element 0.25x0.25 mm mercury cadmium telluride (MCT) detector with high responsivity in the 4000–700 cm\(^{-1}\) region. Data was collected in the 4000-100 cm\(^{-1}\) region although the spectral band-width was limited to the 2000–800 cm\(^{-1}\) region using a band-pass filter. All experiments reported here were undertaken at 2 cm\(^{-1}\) spectral resolution.

A spectrum was taken as an average of 64 scans. The aperture used in this study was 100 \(\mu\text{m}^2\), which was found to yield the sample surface condition. Data were collected from the spectrometer using Varian’s Resolution software, which also computed the ratios of the sample and reference spectra, and then converted the data to absorbance type rather than transmission. Data was then exported in the Data Point Table format.

Every sample mock infected or HSV1 infected had at least 5 spots, and we measured each spot at a minimum of 5 different areas (25 spectra at least). All FTIR experiments reported here were repeated 21 times on independently prepared samples on different days. After the measurements, all the Varian files, which came directly from experiments, were truncated from 1500cm\(^{-1}\) to 800cm\(^{-1}\) and converted into .csv format. All spectra were standardized and then averaged together. Here, standardization is to the process of calculating the mean and standard deviation of a spectrum. The spectrum is then mean centered and divided by the standard deviation.
Standardization is equivalent to vector normalization to within a scale factor. Spectral processing and manipulation were undertaken using software like OPUS 4.0 (Bruker Optics, Billerica, MA), Origin 7.5 (OriginLab Corporation, Northampton, MA), Resolutions Pro 4.05 (Varian Inc., Palo Alto, CA), and Matlab 7.8 (The Mathworks Inc. Natick, MA).

**Results and Discussions**

We have collected spectra for mock and HSV1 infected cellular suspensions at 2, 4 and 6 hours post exposure. Transmission visible images of the samples were similar to those presented previously [53]. Figures 4-1A, 4-1B and 4-1C show vector normalized FTIR spectra for mock and HSV1 infected samples at 2 (A), 4 (B) and 6 (C) hours post exposure (hpe), respectively. Twenty one spectra were collected for each independently prepared mock and HSV1 infected sample. The spectra have been shifted vertically for comparison. Certain spectral trends are observed in nearly all experiments on cells at 2, 4 or 6 hpe. The most noticeable spectral differences are around 1243 cm\(^{-1}\) and 1083 cm\(^{-1}\). In these regions the intensity for HSV1 spectra are mostly higher than mock spectra.

To better visualize the spectral differences between the mock and HSV1 infected cells at the different time points Figures 4-2A, 4-2B and 4-2C show the “HSV1 minus mock” FTIR difference spectra for the 2, 4 and 6 hpe data, respectively. Figures 4-2A, 4-2B and 4-2C demonstrate considerable inter-experimental spectral variation. To more easily visualize the similarities and differences in the spectra at 2, 4 and 6 hpe, Figure 4-2D shows the average of the 2 hpe (yellow), 4 hpe (green) and 6 hpe (pink) “HSV1 minus mock” FTIR difference spectra. Figure 4-2D demonstrates differences in the spectra for HSV1 infected cells at 2, 4 and 6 hpe throughout the
whole spectral region, particularly so in the 1400-1300 cm\(^{-1}\) and 1169-989 cm\(^{-1}\) regions, and below \(\sim 889\) cm\(^{-1}\). The black line is equal to zero difference.

Figure 4-3 compares the averaged difference spectra for all the infection time points from 2 to 48 hpe. 2 hpe (yellow), 4 hpe (green), 6 hpe (pink), 8 hpe (blue), 12 hpe (red), 24 and 48 hpe (black) are all marked in different colors. As was mentioned previously, we have observed the very beginnings of cytopathic effects in the monolayers in visible images of mock samples at 24 hpe [54]. The dramatic differences between spectra from long time exposure (24 and 48 hpe) and short time exposure (2 to 12 hpe) proved that the cytopathic effects have been observed in spectra for 24 and 48 hpe. It is very noteworthy that 1243 cm\(^{-1}\) and around 1000 cm\(^{-1}\) are very useful features for differentiating the 2 and 4 hpe from the other time points.

Visual inspection of the spectra reveals some differences between the spectra of mock infected samples at the range from 2 to 48 hpe. There are some very clear trends in the spectra in Figure 4-1. For Figure 4-2 and Figure 4-3, only if spectra are always collected for mock and infected samples together, some clear conclusions can be derived from the “HSV1 minus mock” FTIR difference spectra (DS). Firstly, for cells at 48 and 24 hpe, the FTIR DS display a negative intensity at \(\sim 1056\) and \(\sim 1227\) cm\(^{-1}\), and a positive intensity at \(\sim 1450\) and 1424 cm\(^{-1}\). These therefore appear to be very good wavenumbers for distinguishing spectra from mock versus HSV1 infected samples at 24 and 48 hpe, at an MOI of 10.

From the FTIR DS in Figure 4-2D, if a positive signal is observed over the whole of the 1300-1500 cm\(^{-1}\) region, then we are likely dealing with HSV1 infected cells that are near 24 or 48 hpe (at MOI=10). Furthermore, as can be seen in Figure 4-2D and Figure 4-3, a trend of decreasing intensity of the difference spectra between HSV1 and mock can be detected as the time post-infection decreases from 48 to 2 hpe.
The FTIR DS in Figures 4-2A to 4-2D indicate that by far the best region for monitoring the progression of HSV1 infection in Vero cells is in the ~1100-1000 cm\(^{-1}\) spectral region. The most surprising feature is that the FTIR DS changes sign for cells at 2, 4, 6, 8 and 12 or 24 hpe. From Figure 3, around 1100 cm\(^{-1}\) also appeared to be a very significant area.

FIGURES

Figure 4-1 Vector normalized FTIR spectra for mock and HSV1
Figure 4-2 “HSV1 minus mock” FTIR difference spectra at 2, 4 and 6 hpe
Figure 4-3 Averages of the “HSV1 minus mock” FTIR difference spectra at 2-48 hpe
5 CHAPTER FIVE T-CELLS ANALYSIS

5.1 Apoptosis In Acute Lymphoblastic Leukemia T-Cells Detected Using Infrared Microscopy

**Keywords:** FTIR, Infrared Microscopy, T-cells, Apoptosis.

**Abbreviations:** A3 T-cells, cells that are a subclone of the Jurkat T-cell line; ALL, acute lymphoblastic leukemia; anti-Fas, anti-Fas; AV, annexin V; AV-FITC, AV-fluorescein isothiocyanate; AUC, area under the ROC curve; CV, cross validation; FACS, fluorescence activated cell sorting; FTIR, Fourier transform infrared; LOOCV, leave one out cross validation; LDA, linear discriminant analysis; PLSR, partial least squares regression; PCA, principle component analysis; PS, phosphatidylserine; PI, propidium iodide. ROC, receiver operating characteristic;
Abstract

We have used FTIR microscopy to study apoptosis induction in acute lymphoblastic leukemia T cells. Apoptosis was induced in the T cells in three different ways: 1) biochemically, by incubating cells in the presence of the anti-Fas antibody, 2) physically, by irradiation of the cells with UVC light, and 3) using hyper-osmotic shock by incubating cells in 1 M sorbitol. Following treatment and then a short four hour incubation period, early stage apoptosis was found to be induced in 70-80% of the cells, using all three methods.

Induction of apoptosis in T-cells gave rise to many changes in the FTIR spectra of dried cellular suspensions, the most prominent being in the 1100-1000 cm\(^{-1}\) region. Using the intensity of a band at 1086 cm\(^{-1}\) it is possible to visually distinguish spectra of control samples from sorbitol or anti-Fas treated cells, which in turn can also be distinguished from the spectra of UVC irradiated cells.

Considerable variability is observed in the spectra of cellular samples, both within one experiment and between several repeat experiments. To quantify how well the spectra for different cells treatments could be discriminated in the face of both types of spectral variability, partial least squares regression analysis in combination with stringent cross-validation methods were used. The statistical analysis procedure developed indicates that the spectra for anti-Fas/sorbitol treated cells are well differentiated from the spectra of control cells and, for a sensitivity of 80%, a specificity of 0.926/0.842 is calculated, respectively. In addition, spectra for anti-Fas/sorbitol treated cells are well differentiated from the spectra of UVC irradiated cells and, for a sensitivity of 80%, a specificity of 0.657/0.789 is calculated, respectively. The FTIR data support the idea that the apoptotic pathways are similar in anti-Fas and sorbitol treated cells, but different in UVC irradiated cells.
Introduction

Apoptosis, or programmed cell death, was first described by Kerr and colleagues [55] as “a basic biological phenomenon with wide-ranging implications in tissue kinetics.” Indeed, extensive studies of this phenomenon established its major role in maintaining normal homeostasis of tissues. Defective apoptosis pathways have been linked to many pathological conditions, such as cancer, autoimmune disease, neurodegenerative disease, viral infection and other pathophysiological states [56, 57].

Apoptosis is mediated by a family of cysteine proteases called caspases. Those associated with apoptosis are expressed in inactive forms (procaspases) in cells and are divided into two classes: initiator caspases (caspase-2, -8, -9, and -10) and effector caspases or executioners (caspase -3, -6, -7). These caspases are engaged by two different mechanisms called the extrinsic and intrinsic pathways. The extrinsic pathway is initiated by ligation of transmembrane death receptors (Fas, TNF and TRAIL receptors), resulting in recruitment and activation of caspase-8 and -10 which then cleave and activate effector or executioner caspase 3 or 7. Intrinsic pathways require disruption of mitochondrial membranes resulting in release of cytochrome-c, which works together with apoptotic protease activating factor-1 (Apf-1) and procaspase-9 to bring about the formation of a caspase-activating complex called an apoptosome. This results in the activation of caspase-9 to initiate the apoptotic caspase cascade. In some cases both extrinsic and intrinsic pathways can be engaged simultaneously [58].

Cells undergoing apoptosis exhibit major morphological and biochemical changes. Morphological changes include membrane blebbing and cell shrinkage. Biochemical changes include chromatin condensation and DNA fragmentation (ladder formation) [59], changes in protein synthesis [60] and translocation of phosphatidylserine (PS) from the cytoplasmic side of the plasma
membrane to its outer side, exposing PS to the medium [61]. Although the latter may be caused by non-apoptotic stimuli, it is a consistent marker present during apoptosis [62].

Acute lymphoblastic leukemia (ALL) is a neoplasm of lymphocytes characterized by a clonal accumulation of immature blood cells (lymphoblasts) in the bone marrow. The cells involved can be from B or T lineage and they can invade any organ in the body. Major tissues involved include lymph nodes, spleen, liver, central nervous system and skin. Two distinct forms of ALL are recognized, the childhood and the adult diseases, and usually the first has a better survival rate. Treatment of ALL involves chemo, immune, and/or radiotherapy aimed at inducing apoptosis in rapidly dividing cells. From an understanding of the core components of the apoptosis machinery at the molecular and structural levels, potential new therapies for leukemia and lymphoma are emerging [63]. With knowledge of apoptosis-induced Fourier transform infrared (FTIR) spectral biomarkers, it may be possible to assess how malignant cells respond (via apoptosis) to some of these new therapies prior to the initiation of individual treatment.

There is considerable clinical utility in being able to quantify the extent of cellular apoptosis for a number of disease states, including leukemia [56, 64]. In recent years the unique capabilities of FTIR spectroscopy to study molecular processes in the cell have become more evident [16, 43]. In particular, FTIR spectroscopy is a nondestructive technique that may allow one to rapidly obtain information on a variety of macromolecules inside cells. Given this, in this manuscript we report on the use of FTIR spectroscopy to monitor apoptosis induction in ALL cells.

Apoptosis can be induced in cells in a variety of ways and in this manuscript we report on three different methods used to induce apoptosis in a Jurkat T-cell subclone. Firstly, we biochemically induced apoptosis in cells by incubating them in the presence of the anti-Fas antibody. Fas (CD95) is a cell surface protein that is a member of the tumor necrosis factor (TNF) re-
ceptor super-family. Its activation, via binding to the Fas-L ligand or antibody, results in the trimerization of Fas and recruitment of FADD (Fas-associated protein with death domain) [65]. FADD recruits pro-caspase-8, which initiates activation of caspase 8 and the subsequent caspase cascade that leads to apoptosis (the so-called extrinsic pathway) [65].

A second (physical) approach we used to induce apoptosis is to irradiate cells with UVC light [58, 66, 67]. UVC light can induce apoptosis by activation of p53 and death receptors expressed on the cell surface via release or upregulation of death ligands. Death receptor activation is ligand-independent and occurs via receptor clustering. Whether the UVC-induced DNA damage occurs first or in parallel to these events is unknown [66, 67].

A third approach used was to induce hyper-osmotic shock by incubating cells in 1 M sorbitol [68]. The stress response that is induced leads to activation of a family of p21Cdc42/Rac-activated kinases (PAKs), inducing cleavage of PAK2 [69] which is closely associated with both DNA fragmentation and activation of caspase 3, an executioner caspase, resulting in cell death via apoptosis.

FTIR spectroscopy has been used to study apoptosis induction in cellular samples previously [70-72]. In most of these studies, however, the extent of inter-experimental spectral variability that could occur for cellular samples was not factored into the analyses. Without considering inter-experimental spectral variability it is difficult to unambiguously assess and quantify apoptosis-specific changes.

In many previous FTIR microscopy studies of different cell types, the spectra were analyzed using statistical modeling. The results obtained from the modeling usually overestimate the capabilities of FTIR spectroscopy in distinguishing cells in different states, however. This overestimation arises because the statistical models are poorly validated. Improper validation of sta-
istical models can occur because inter-experimental spectral variability is not considered. For example, it is common in FTIR spectroscopy of cellular samples to collect many spectra for each sample, and then repeat the experiment several times. All of the spectra from each of the experiments are then statistically analyzed simultaneously. To assess the level of sensitivity and specificity obtainable from the spectra from different cell types some type of cross validation is required - usually leave-one-out-cross-validation (LOOCV) is used [30]. However, LOOCV inadequately accounts for inter experiment spectral variability that is common in FTIR microscopic measurements of cellular samples. One consequence of this is that spectra of different cell types are distinguished with very high levels of specificity and sensitivity, and the capabilities of FTIR spectroscopy in distinguishing different cell types is greatly overestimated. Or put another way, the calculated specificities and sensitivities do not properly reflect the level of accuracy achievable in blind tests where cell type is established based on the spectra obtained.

In this paper we describe the induction of apoptosis in ALL T-cells using three different methods. The extent of apoptosis induction was monitored using flow cytometry (FACS). FTIR microscopy was then used to obtain spectra for control and apoptotic cells. At least 25 spectra were collected for any one sample, and we have repeated all sample measurements 5-6 times. The spectra were analyzed using partial least squares regression (PLSR) methods. Our ability to discriminate spectra from different cell types was assessed using cross validation methods designed to appropriately account for the inter experimental spectral variability that is observed in our experiments.
Materials and Methods

Cells: In the studies reported here the A3 sub-clone of the ALL Jurkat T-cell line (ATCC CRL 2570) was used. This cell line is particularly sensitive to Fas induced apoptosis (REF). We will refer to these cells as A3 cells in the rest of this manuscript. A3 cells were grown at 37°C in a 5% CO₂ humidified incubator. Cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA), penicillin and streptomycin. Cell cultures were expanded by dilution of cells to a concentration of 3-5\times10^5 cells/ml and subsequently split every two to three days to maintain cell counts.

Induction of apoptosis. For anti-Fas induced apoptosis 4-5 ml of cells (0.7-1\times10^6 cells/ml) in the logarithmic phase of growth were centrifuged for 5 min. at 1300 rpm and resuspended to the same concentration with RPMI without FBS. Anti-Fas antibody (500 ng/ml) (clone CH11, 0.5 mg mouse IgM/ml) (Millipore, USA) was added to the cells, which were then incubated at 37°C in 5% CO₂ for 4 hours.

For sorbitol induced apoptosis, cells (0.7-1\times10^6 cells/ml) were centrifuged as above and resuspended with 4-5 ml of RPMI supplemented with 2% FBS containing 1 M sorbitol. Following 1 hr. incubation at 37°C in 5% CO₂ cells were centrifuged again and resuspended to the same cell concentration with RPMI supplemented with 10% FBS. Cells were then incubated for additional 3 hours. Control cells were treated identically except without sorbitol.

For UVC-irradiation induced apoptosis, cells (0.7-1\times10^6 cells/ml) were transferred to 6-well plates at 0.8 ml cells per well. The cells were placed at a distance of 30 cm from a 25 W Philips germicidal lamp emitting at 254 nm, and irradiated for 30 seconds. The corresponding dose is 250 J/m², which was monitored using a General UV512C light meter (General Tools and
Instruments, NY). Control cells were treated identically but without the UVC irradiation. Cells were then incubated at 37°C in 5% CO₂ for an additional 4 hours.

Assessment of apoptosis by flow cytometry. Following induction of apoptosis and incubation, cells were centrifuged as above, washed once with PBS and labeled with the Annexin-V-Fluos staining kit (Roche, USA) according to the manufacturer instructions. Labeled cells were then analyzed by fluorescence activated cell sorting (FACS) using a FACS Aria Cell-Sorting System (BD Biosciences, San Jose, CA). Propidium iodide (PI) in the kit allowed assessment of the fraction of early and late apoptotic cells, as well as differentiation from necrotic cells.

Detection of DNA fragmentation by agarose gel electrophoresis. For DNA fragmentation analysis cells were lysed and cellular DNA was extracted and fractionated electrophoretically on a 1.5% agarose gel electrophoresis. Gel patterns were analyzed using ethidium bromide staining.

Preparation of cellular material for FTIR measurements. For (transflection) FTIR microscopy measurements, following induction of apoptosis and incubation, cells were centrifuged as above, washed once with PBS and once with saline. Cells were then resuspended with 15-20 l of saline diluted 1:5 (to avoid formation of salt crystals). Small drops of cells were then spread on MirrIR reflective windows (Kevley Technologies, Chesterland, OH) and allowed to air dry for 5-10 min. at room temperature. FTIR measurements were then undertaken immediately.

FTIR Microscopy Instrumentation and Parameters. FTIR microscopy measurements were undertaken using a Varian 7000 FTIR spectrometer (Varian, Lake Forest, CA) coupled to a Varian UMA600 IR microscope. The microscope was setup in reflection mode (transflection) using a 15X Schwartzchild objective. The microscope was equipped with a visible light source and CCD camera that allows the collection of visible microscopic images from the same sample region that is investigated in the IR. The IR light was detected using a single element 0.25x0.25
mm mercury cadmium telluride (MCT) detector with high responsivity in the $4000$–$700\ \text{cm}^{-1}$ region. Data was collected in the $4000$-$100\ \text{cm}^{-1}$ region although the spectral bandwidth was limited to the $2000$–$800\ \text{cm}^{-1}$ region using a band-pass filter. All experiments reported here were undertaken at $4\ \text{cm}^{-1}$ spectral resolution.

Several cell suspension “spots” were pipetted onto a MirrIR slide and air-dried. The suspension spot has a diameter of $\sim 3\ \text{mm}$. For microscopic measurements the area sampled is $\sim (100 \ \mu\text{m})^2$. For each set of “spots” (usually 3-5) spectra were collected at about 25 different locations on the spots. Each spectrum collected was the co-addition or average of 64 interferograms. All FTIR experiments reported here have been repeated five or six times on independently prepared samples on different days. We have found that FTIR spectra collected near the edges of the sample spots display less variation than spectra obtained near the center of the spots. In all FTIR spectroscopy measurements the IR absorption in the amide I region was $\sim 0.8$-$1.2$ in optical density units.

*Spectral Analysis.* All spectra were first truncated to consider only the $1800$-$800\ \text{cm}^{-1}$ region, or the $1500$-$800\ \text{cm}^{-1}$ region, and then standardized. For standardization it is necessary to calculate the mean and standard deviation of a spectrum. The spectrum is then mean centered and divided by the standard deviation. Standardization is equivalent to vector normalization to within a scale factor. Second derivative spectra were calculated without smoothing. Spectral processing and manipulation were undertaken using OPUS 4.0 (Bruker Optics, Billerica, MA), Origin 7.5 (OriginLab Corporation, Northampton, MA), Resolutions Pro 4.05 (Varian Inc., Palo Alto, CA), Matlab 7.8 (The Mathworks Inc. Natick, MA) software. All statistical analysis of the spectra reported here was undertaken using home-written code implemented within the SAS (Cary, NC, USA) software programming environment.
Results

*Flow cytometry assessment of apoptosis induction in ALL T-cells.* We have employed three different methods to induce apoptosis in A3 cells (Jurkat T-cells, A3 subclone), and we have used flow cytometry to monitor the extent of apoptosis in the cell population. Figure 1A shows scatter plots derived from flow cytometry measurements on normal cells, and cells where apoptosis was induced by each of the three different methods.

For UVC irradiation of A3 cells it was found that 30 seconds (at 30 cm from light source) was the optimal irradiation time for induction of apoptosis. Longer irradiation times of 45 or 60 seconds had no further effect on the percentage of early apoptotic cells (not shown). With the anti-Fas treatment the antibodies were present in the medium during the entire incubation period. For the hyperosmotic shock method using sorbitol, the exposure period with sorbitol was 1 hr. For each method used the incubation period following the beginning of treatment was 4 hrs.

For each of the three procedures used to induce apoptosis, for an incubation period of 4 hours, the majority of the cells (78.3-83.7%) were found to be in the early apoptosis phase (Figure 1A). The population of cells showing late-stage apoptosis was very low (2.7-4.4 %) (FITC-Annexin positive/propidium iodide positive), as was the necrotic fraction (0.4-1.5 %). It should be noted that measurements of late-stage apoptosis can include necrotic cells as well. The data show that the percentage of apoptotic cells was comparable for each of the three methods used. In addition, the extent of DNA ladder formation was also comparable (Figure 1B).

*Comparison of FTIR spectra from control and anti-Fas treated cells.* Figure 2A shows raw spectral data collected for control (black) and anti-Fas (red) treated A3 cells. For control and antibody treated cellular suspensions, spectra were collected at 23 and 26 locations on the sample “drops”, respectively. The data in Figure 2A demonstrates variation in the intensity of the spectra
collected at various locations on the dried cellular suspensions. The variation in spectral intensity is similar in magnitude to that found previously for Vero cell suspensions [44]. To eliminate any effects on the spectra that may be due to sample thickness, or changes in cellular concentration, the spectra in Figure 2A were standardized. The calculated standardized spectra are shown in Figure 2B.

Although considerable variation in the spectra collected from the same sample still exists, Figure 2B indicates that there are spectral regions where the spectra of anti-Fas treated cells can, for the most part, be distinguished from spectra of control cells. In particular, most of the spectra for anti-Fas treated cells display decreased absorption in the 1000-1100 cm\(^{-1}\) region, and increased absorption in the 1800-1700 cm\(^{-1}\) region, compared to spectra of control cells. Figure 2B demonstrates that the spectra for control and anti-Fas treated cells are not easily distinguished from consideration of the amide I and II absorption bands (1680-1620 and 1560-1500 cm\(^{-1}\) regions).

To better visualize the differences in the spectra of control and anti-Fas treated cells the average spectra for control and anti-Fas treated cells are shown in Figure 2C. To gauge the variation in the standardized spectra the error bars are also shown in Figure 2C. These error bars represent the standard deviation in the spectra for the 23 or 26 spectra in Figure 2B. Figure 2D shows the same spectra as in 2C without the error bars.

The data in Figure 2C demonstrate that there is no overlap of the error bars in most of the 1780-1700 and 1130-1030 cm\(^{-1}\) regions. These two spectral regions are therefore useful indicators of anti-Fas induced apoptosis in A3 cells. The averaged spectra shown in Figure 2D also indicate that the whole of the 1300-900 cm\(^{-1}\) region spectra of anti-Fas treated cells display decreased absorbance compared to that in spectra of control cells. In the 1480-1430 cm\(^{-1}\) region,
absorption for anti-Fas treated cells is increased relative to control cells. Although there is some overlap in the error bars in these spectral regions, these regions may still be useful for discrimination, albeit with somewhat lower sensitivity than the 1300-900 cm$^{-1}$ region.

The data in Figure 2A-D is for a single experiment, for cells prepared on a single day. It is important to quantify the extent of inter-experimental spectral variation, and Figure 2E shows the averaged FTIR spectra of control and anti-Fas treated A3 cells obtained in five independent experiments. The changes in the spectra in Figure 2C or D described above were also reproducibly observed in the five independent experiments. For example, at the frequencies marked by the dotted lines in Figure 2E the spectra for the control and anti-Fas treated samples display the same trends in all five experiments. That is, in all five experiments, spectra for control cells display an increased absorption at 1656, 1240, 1086 and 966 cm$^{-1}$ and a decreased absorption at 1744, 1540 and 1466 cm$^{-1}$, relative to spectra for anti-Fas treated A3 cells.

Qualitatively, anti-Fas treated A3 cells can be discriminated from control cells based on the above spectral features. However, our goal is a quantitative and robust procedure to ascertain what levels of sensitivity and specificity are achievable from our spectral measurements. Such an analysis is presented below.

Comparison of FTIR spectra from all cell types. Figure 3A-C compares the averaged spectra from five or six independent measurements on cells that have been exposed to UVC light (blue), sorbitol (magenta) or anti-Fas (green), as outlined in the materials section. The absorbance spectra were first standardized and then averaged in each independent experiment. The results of the six averages are then further averaged to produce the final spectra shown in Figure 3. FTIR measurements on two different types of control cells are also shown in Figure 3. One set of control cells is for the UVC treated cells while the other is for sorbitol and anti-Fas treated
cells. The expectation is that spectra for the two types of control cells should be similar. This is indeed found to be the case. Also shown in Figure 3 are the calculated, unsmoothed second derivative spectra. Five absorbance difference spectra, that represent the subtraction of one spectrum from another, are also shown.

In Figure 3 the original absorption spectra were standardized. However we have also considered the same spectra normalized in different ways (for example we have normalized the spectra so that the maximum and minimum absorbance values are the same), and we have found that similar difference spectra, with peaks at similar frequencies to that shown in Figure 3, are obtained (not shown). Therefore, the features (peaks and dips) in the absorbance difference spectra in Figure 3 are not related to the normalization process.

Statistical analysis. Inspection of the experimental spectra demonstrates considerable variation both within experiments on any given day (Figure 2A), and between measurements on different days (Figure 2E). This variation persists in spite of the fact that sample preparation and FTIR experiments were undertaken in an identical fashion. This intra and inter-experimental spectral variability obviously limits our ability to distinguish spectra from control and treated cells. Any statistical methods and data processing procedures developed should take steps to minimize and account for both intra and inter experimental spectral variability. For analysis of our spectral data we have developed the following procedures:

1 Spectra are truncated to select the 1500-800 cm\(^{-1}\) region, and then standardized. For initial studies we do not consider the amide I and II spectral regions (1800-1500 cm\(^{-1}\)). This approach is taken to avoid any potential dispersion and other scattering induced artefacts that contribute most to spectra in the amide I and II spectral regions.
In each spectrum groups of five neighboring data points are averaged. This reduces each spectrum to 73 data points (variables). This procedure reduces the quantity of data to be analyzed. It also increases the robustness of the statistical method.

The 20-30 spectra collected for any given sample in any given experiment are split randomly into two groups of 10-15 spectra, and the mean spectrum for both groups is calculated (this averaging helps reduce intra experimental spectral variability). For example, for spectra obtained using control and anti-Fas treated T-cells in any given experiment, four averaged spectra are produced, which can be labeled (for convenience) as control₁, control₂, anti-Fas₁ and anti-Fas₂.

From the four spectra obtained in step 3, two statistics are generated: One statistic is the difference between the two averaged spectra from the same sample [(control₁ minus control₂) and (anti-Fas₁ minus anti-Fas₂)]. We will refer to these spectra as inner difference spectra. The second statistic is the difference between the control and treated samples [(control₁ minus anti-Fas₁) and (control₂ minus anti-Fas₂)]. We will refer to these spectra as intra difference spectra. Note that the intra difference spectra (control₁ minus anti-Fas₂) and (control₂ minus anti-Fas₁) are not considered as they are correlated with the other two intra difference spectra.

The inner difference spectra provide a baseline from which to consider intra difference spectra. This approach was used to help minimize effects due to inter experimental spectral variability.

Since we have collected spectra for control and treated T-cells in 5-6 different experiments, we have 10-12 inner- and intra-difference spectra. These inner and intra-difference spec-
tra are shown in Figure 4A for control and anti-Fas-treated cells. Similar figures can be generated for all other paired comparisons (not shown).

5 In order to establish which frequencies in the intra difference spectra are of highest diagnostic value the Wilcoxon signed rank test (WSRT) was applied to the inner and intra difference spectra. The WSRT is a one sample test \[48\], which is appropriate since we are interested in variables in the intra difference spectra that differ significantly from zero.

Figure 4B shows a plot of the p-values at each frequency calculated using the WSRT. Bonferroni’s multiple comparison method\[73\] suggests that frequencies with p<0.00068 should be highly significant. In practice, only a few of the frequency variables have such low p-values, so frequency variables with higher p-values were considered (see Table 2). From the p-value spectrum in Figure 4B, we find 31 frequencies with p<0.003 (see inset). Only these 31 frequencies are used in the construction of a discriminating statistic.

6 Partial least square regression (PLSR) \[74\] was then applied using the significant frequency variables. As described previously \[46\], to evaluate the performance of our PLSR model in correctly classifying any two categories of spectra, specificities and sensitivities were calculated using the constructed PLSR model. This allowed a receiver operating characteristic (ROC) curve to be constructed, and the area under the ROC curve (AUC) was then calculated. Sensitivity gives a measure of the proportion of correct identifications among actual positives, while specificity gives a measure of the proportion of correct identifications among all negatives. A ROC curve is a plot of sensitivity vs. (1-specificity). A calculated AUC of 1 represents 100% accuracy of discrimination. A rough classification guide is: AUC = 0.9–1.0, excellent discrimination; AUC = 0.8–0.9, good discrimination; AUC = 0.7–0.8, fair discrimination; AUC < 0.6–0.7, poor discrimination.
Table 1 lists the results of PLSR analysis applied to the spectra (significant frequencies) for control and anti-Fas treated cells. For a sensitivity of 95%, a specificity of 1.0 is calculated. The AUC is also calculated to be 1.0. This indicates perfect discrimination of the inner and intra difference spectra for control and anti-Fas-treated cells.

In PLSR analysis, calculated specificities (for given sensitivities) are generally overestimated. As a first step in “estimating this overestimate” we have used two-fold cross-validation [75]. In this procedure, half the data is selected randomly, and used to build the statistical model. The constructed model is then applied to the other half of the data. We repeated the two-fold cross validation procedure fifty times. The results presented in Table 1 are the average.

Given the small sample size (12 spectra), large variations in the calculated specificities are expected in the model applied to the validating data. That is, a large shrinkage is expected. Shrinkage is the difference in the specificity calculated between the validating data and the original data. Table 1 lists the values for specificity, sensitivity and AUC calculated using two-fold cross-validation. For a sensitivity of 95/90/80 %, a specificity of only 0.42/0.58/0.77 was obtained, and the calculated shrinkage was 0.58/0.42/0.22, respectively. Using 2-fold cross-validation the AUC drops to 0.868, which still represents good discrimination capability. It is not at all clear whether the discrimination capability will increase if further experiments are undertaken and included in the analysis.

Two-fold cross-validation [27,62,65,67] methods generally overestimate shrinkage. In a second approach we also used Bootstrap methods [76] to estimate shrinkage. Using Bootstrap methods for a sensitivity of 95/90/80 %, a specificity of 0.90/0.95/0.99 was calculated, along with a shrinkage of 0.1/0.05/0.01, respectively (Table 1). The calculated AUC was 0.983, which represents excellent discrimination capability.
Bootstrap calculated shrinkage is usually an under-estimate of the true value. A useful rule of thumb is to use the average shrinkage calculated using the two different validation procedures. The final specificities and AUC calculated by subtracting the initial model values from the averaged shrinkage are also listed in Table 2. The calculated AUC after accounting for shrinkage is 0.926, which represents a quite outstanding level of discrimination achievable from spectra of control and anti-Fas treated cells.

The statistical analysis outlined above for control and anti-Fas treated T-cells was also undertaken for all pairs of combinations of the five sets of cells (control, control UV, UV treated, anti-Fas treated and sorbitol treated cells). The calculated AUC and specificity for 80 % sensitivity from these sets of analysis are listed in Table 2. Also listed are the cut-off p-values used in two-fold cross-validation and Bootstrap analyses.

Several points from the data in Table 2 are noteworthy: 1) The spectra from anti-Fas and sorbitol treated cells could not be discriminated. This is not surprising, as the spectra for the two samples were found to be very similar (Figure 3). 2) Somewhat surprisingly, the spectra from control UV (or control) cells could not be discriminated from spectra of UV treated cells. 3) Spectra obtained for anti-Fas and sorbitol treated cells were easily distinguished from spectra of control (or control UV) cells. 4) Spectra obtained for anti-Fas and sorbitol treated cells were distinguishable from spectra of UV treated cells.
Discussion

Many anti-leukemic or lymphoma therapies are aimed at inducing or restoring apoptosis in malignant cells. From an understanding of the core components of the apoptosis machinery at the molecular and structural levels, many potential new therapies for leukemia and lymphoma are emerging [63]. With knowledge of apoptosis-induced spectral biomarkers one may be able to (rapidly) assess how malignant cells respond (via apoptosis) to some of these new therapies. Thus a FTIR detection technology, together with knowledge of spectral biomarkers of malignant lymphocytes, may be used as a new complementary tool for rapidly testing drug resistance in leukemic cells directly isolated from patients. Indeed the use of FTIR spectroscopy to monitor leukemia progression in real patients has already begun [77, 78]. For such work to be fruitful a detailed understanding of FTIR spectral alterations that occur upon apoptosis in cells is required.

It has previously been established that most of the bands in the FTIR spectra of cellular samples contain contributions from many sub-cellular components [79]. Many sub-cellular components have been isolated and their FTIR spectra have been obtained. From knowledge of these spectra of isolated components it can then be inferred which components may contribute to the bands in the FTIR spectra. Note that when a FTIR band changes, due to for example apoptosis induction, it may be suggested that the change is related to a given sub-cellular component. This is not a proof, however. An important realization in recent years is that scattering effects contribute to spectra of cellular suspensions. Therefore, it should be kept in mind that changes in scattering upon apoptosis induction may be at the heart of some of the spectral changes observed, rather than changes in concentration of sub-cellular components.

With these caveats in mind it is still worthwhile indicating which sub-cellular components may contribute to the bands in the FTIR spectra in Figure 3. Lipid ester carbonyl (C=O)
vibrations are known to occur in the 1750-1710 cm$^{-1}$ region [54, 80, 81]. The weak bands at 1744 and 1716 cm$^{-1}$ in the spectra in Figure 3A can be associated with (phospho) ester lipids [54]. The asymmetric and symmetric bending of lipid methyl and methylene groups is known to occur near 1460 and 1400 cm$^{-1}$ [82-85]. The bands that occur near 1456 and 1398 cm$^{-1}$ in the spectra in Figure 3B may therefore contain contributions from these species.

The asymmetric and symmetric vibrations of the phosphodiester groups of DNA occur near 1240 and 1085 cm$^{-1}$ [84]. The bands at 1241 and 1086 cm$^{-1}$ in the spectra in Figure 3B and C may therefore contain contributions from these DNA modes. The band at 966 cm$^{-1}$ in Figure 3C may contain contributions from the vibration of the C-C/C-O conglomerate in DNA [84].

From examination of the spectra in Figure 3, several features are noteworthy:

First, the spectra for the two types of control samples are very similar (compare black and red spectra in Figure 3). This is as expected.

Second, bands at 1744 cm$^{-1}$ and 1716 cm$^{-1}$ are observed in the spectra of all five samples. As mentioned above, these bands are likely associated with ester lipids. Although the apoptosis induced changes in these bands are weak, it appears that sorbitol, anti-Fas and UVC treatment causes a small decrease in the intensity of the 1716 cm$^{-1}$ band, but not the 1744 cm$^{-1}$ band.

Third, for all five samples the amide I band peaks at 1658 cm$^{-1}$ and displays a shoulder at 1639 cm$^{-1}$. These features are readily visualized in the second derivative spectra (Figure 3A). Changes in the amide I band due to apoptosis are apparent in the spectra in Figure 3A: The spectra for the two control samples show an increased intensity peak at 1658 cm$^{-1}$. This gives rise to a negative peak (dip) at 1655-1659 cm$^{-1}$ in all four of the FTIR difference spectra that involve a subtraction of one of the two control spectra. The anti-Fas, sorbitol and UVC light treated samples all appear to have amide I bandwidths that are slightly increased (by ~2 cm$^{-1}$) relative to the
two control samples. The second derivative spectra in Figure 3A indicate that this is because of a small increase in intensity of a band near 1631 cm$^{-1}$ in the spectra of the apoptotic cells. Such changes could be consistent with the transformation of some alpha helical protein content to beta sheet upon apoptosis [86]. However, in studies of cells, interpretation of changes in the amide I band are notoriously difficult to make. This is principally because IR light scattering and dispersion effects all have a large impact on the amide I absorption band [54, 87]. Specifically, apoptotic cells may scatter IR light differently from non-apoptotic cells, which may lead to changes in the amide I band profile. It is because of these types of complications that we chose to exclude the amide I (and II) spectral region from our statistical analysis. However, if one is interested only in identifying spectral biomarkers that can pinpoint apoptosis in A3 cells, then the amide I absorption band may be a useful indicator of apoptosis, at least in experiments following the type of protocol used here.

Fourth, the amide II band peaks near 1545 cm$^{-1}$ and displays a distinct shoulder near 1516 cm$^{-1}$. Again, these features are more easily visualized in the second derivative spectra (Figure 3A). The width of the amide II band appears to be slightly increased in spectra for cells in which apoptosis has occurred. However, again, this does not necessarily reflect changes in protein structure in apoptotic cells relative to control cells.

Fifth, in the 1500-1150 cm$^{-1}$ region, FTIR absorbance difference spectra 1-4 all display negative peaks near 1400 and 1241 cm$^{-1}$ (Figure 3B). These peaks are most pronounced in difference spectra 1 and 2. That is, anti-Fas and sorbitol induced apoptosis each cause a greater decrease in absorption of the 1400 and 1241 cm$^{-1}$ bands (relative to control spectra) than does apoptosis induced in cells via UVC irradiation. This may be an indication that FTIR spectrosc-
py can distinguish cells that have undergone apoptosis via extrinsic and intrinsic pathways, although further work will be required to verify or disprove this statement.

Six, in the 1150-900 cm$^{-1}$ region there are many differences in the spectra obtained from the different sample preparations (Figure 3C). Again, the spectra for the anti-Fas and sorbitol treated samples are very similar as evidence by the lack of features in the anti-Fas minus sorbitol absorbance difference spectrum (spectrum 5 in Figure 3C). The spectra for the two control samples are also very similar, but distinct from the spectra for the anti-Fas and sorbitol treated samples. The spectrum for the UVC treated samples lies between the two extremes. This pattern can be seen in all of the bands in the 1150-900 cm$^{-1}$ region.

Seven, in the FTIR spectra for the five samples shown in Figure 3C the largest changes in bands are found at 1086, 1054 and 1023 cm$^{-1}$. The second derivative spectra in Figure 3C (bottom set of curves) show that all the spectra of cells display a peak at 1086 cm$^{-1}$. The presence of a negative peak (or dip) at 1086 cm$^{-1}$ in the absorbance difference spectra in Figure 3C (middle set of curves) indicate that the peak at 1086 cm$^{-1}$ in the spectra of the control samples decreases in intensity for UVC treated samples, and decreases even further for anti-Fas and sorbitol treated samples.

Eight, the second derivative spectra show a negative feature at 1054-1056 cm$^{-1}$ for all cell samples (Figure 3C). A negative feature is also observed near 1049 cm$^{-1}$ in the second derivative spectra for the control samples, but is absent in spectra of treated (apoptotic) cells. This difference manifests itself as a broad negative band near 1049 cm$^{-1}$ in difference spectra 1, 2 and 4. The data suggest that a band near 1049 cm$^{-1}$ in spectra of control cells is lost upon apoptosis induction.
Comparison to previous studies: FTIR spectroscopy, at both the microscopic and macroscopic levels, has been used to study apoptosis in various types of cells. Much of this work has been summarized in several recent reviews [70-72]. Comparison of the different studies that have been undertaken is difficult, partly because of the differences in the way the experiments were undertaken (different cell sample types and preparation techniques were used, different FTIR techniques were used, different methods for statistical analysis of data were employed, different methods for quantifying the extent of apoptosis induction were used, and in some cases it is not clear whether the cells under study had undergone apoptosis or necrosis).

Given the wide variety of conditions used in different experiments there is considerable confusion in the literature concerning what spectral observables could be altered (and how) upon apoptosis induction in cells. Our studies on cells in the early apoptosis stage (measured at 4 hours post treatment) suggest only small (but reproducible) spectral changes. In contrast, very large spectral changes have been observed upon apoptosis induction in some previous studies [64, 88, 89].

Spectra for anti-Fas and sorbitol treated cells differ from the spectra of cells irradiated with UVC light. Interestingly, our results show that FTIR spectra for cells undergoing anti-Fas and sorbitol induced apoptosis are similar, but clearly distinct from spectra of cells with apoptosis was induced using UVC light. It is not clear if these spectral differences indicate differences in the final apoptotic state of the cells studied. Such differences are not suggested by the FACS data presented in figure 1A.

The Fas signaling pathway, one of the extrinsic apoptosis pathways, involves ligand binding to the Fas receptor on the cell surface, followed by recruitment of death domain-containing
FADD and procaspase-8 [60] and subsequent activation of the caspase cascade, p38 MAP kinase and JNK [90].

In the intrinsic pathway cytochrome c is released from the mitochondria and subsequently interacts with Apf-1 and procaspase-9 to promote the assembly of the apoptosome to start a cascade that leads to cell death. The general dogma for UV and sorbitol induced apoptosis is that in both cases the intrinsic pathway is involved. However, since death domain recruitment due to ligand-independent receptor clustering can occur with sorbitol, it is likely that activation of the extrinsic pathway dominates, driving apoptosis associated with hyperisomotic shock. It has been reported that UVC light can induce apoptosis in mouse embryo fibroblast via both intrinsic and extrinsic pathways [58]. Several further reports have also shown that DNA damaging agents, such as UV radiation or drugs used for chemotherapy, induce apoptosis via the Fas/caspase 8 pathway [91-94]. Different treatments may lead to apoptosis induction in cells via different contributions from intrinsic and extrinsic pathways. However, it is quite unclear if this could give rise to spectral differences in the differently treated cells. More detailed and focused studies will be required to address this question.

Statistical Analysis. A crucial feature of the statistical analysis presented here is that the data on different days are treated as independent single samples. Thus we have shown quantitatively that spectral discrimination between differently treated T-cells is possible, even in the presence of considerable inter experimental spectral variability.

In a recent FTIR microscopy study of different lymphoma and leukemia cell types, a principle component fed linear discriminant analysis (PC-LDA) in combination with leave-one-out cross validation (LOOCV) approach was used to discriminate the spectra of the different cell types [30]. The levels of sensitivity and specificity achieved using this PC-LDA LOOCV ap-
proach were very high [30]. We have analyzed the spectral data presented here using the same PC-LDA LOOCV approach, and we also obtain similarly high levels of sensitivity and specificity for distinguishing spectra of differently treated cells (not shown). The levels of sensitivity and specificity we achieved using the PC-LDA LOOCV approach are much higher than that achieved using the statistical methods for cross-validation outlined here. This is because the LOOCV approach does not properly account for inter-experimental variability: LOOCV uses one spectrum from all the spectra collected over many experiments as the validation data, and the remaining spectra are used as training data. This is the same as K-fold cross-validation [75], with K being equal to the total number of spectra. If the data collected over different experiments is consistent then similar results from K-fold cross-validation and LOOCV should be obtained. However, we obtain considerably lower sensitivities and specificities (than that obtained using LOOCV) when we undertake cross validation with all of the spectra for one experiment are used as the validating data (six-fold cross validation in our case). This observation indicates that interexperimental spectral variability is a problem that is not properly accounted for using the LOOCV approach.

One of the main goals of FTIR spectroscopy in studies of cells is to be able to accurately classify spectra according to specific cell type. For example, in the cases studied here, if we are given spectra from all five cell types, without knowledge from which cell type the spectra are collected, how well are we able to classify the spectra? We have found that even although we calculate very high levels of specificity and sensitivity for distinguishing spectra of different cell types using the LOOCV approach, we have a much lower accuracy in successfully classifying unknown spectra to a given cell type using LOOCV. The regression analysis approach outlined
in this manuscript gives a more accurate and realistic measure of how well we can classify spectra without prior knowledge from what cell type the spectra came from.

In summary, few FTIR studies of cells in different states have included an assessment of inter experimental spectral variability. The methods used to calculate sensitivity and specificity for distinguishing spectra from different cell types in many previous studies do not accurately reflect how well FTIR spectra from unknown cell types can be classified.

Acknowledgements

This work was supported by the Department of the Army (U.S. Army Medical Research Acquisition Activity, Contract grant number: W81XWH-06-1-0795, and the Georgia Research Alliance. The opinions or assertions herein are the private views of the authors and are not to be construed as reflecting the views of the Department of the Army or the Department of Defense.
References


Table Legends

Table 1: Results of PLSR analysis applied to spectra from control and anti-Fas treated cells collected from six different experiments. Two fold cross validation and a bootstrap method were used to estimate shrinkage, and provide a realistic estimate of obtainable sensitivities and specificities. For the two fold cross validation /bootstrap method data points with p-values less than 0.05/0.003 were chosen for analysis.

Table 2: Results of PLSR analysis applied to spectra of different pairs of cell types. The results listed are obtained anti-Fas faster including shrinkage that was estimated using 2 fold cross validation and Bootstrap methods. The items listed in each cell are, from top to bottom: AUC, level of discrimination, specificity for 80% sensitivity and p-value used in bootstrap/two fold cross validation methods. Empty cells indicate no discrimination.
### Table 1

**Control versus anti-Fas treated cells**

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<th>Specificity (CV)</th>
<th>Shrinkage (CV)</th>
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### Table 2

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<td></td>
<td>Good</td>
<td>Good</td>
<td>Poor</td>
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<td>0.713</td>
<td>0.782</td>
<td>0.789</td>
<td></td>
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<tr>
<td></td>
<td>0.003/0.3</td>
<td>0.003/0.2</td>
<td>0.04/0.2</td>
<td></td>
</tr>
</tbody>
</table>
Figure Legends

**Figure 1:** (A) Flow cytometry data obtained for untreated A3 cells (Jurkat T-cells, A3 subclone3) *(top left)*, and A3 cells in which apoptosis has been induced using anti-Fas (500 ng/ml) *(top right)*, sorbitol (1M for 1 hr) *(bottom right)* and UVC irradiation (30 sec exposure) *(bottom left)*. Cells were incubated for 4 hrs post treatment. FITC+ corresponds to early apoptotic cells; PI+/FITC+ correspond to late apoptotic cells; PI+ correspond to necrotic cells. (B) Agarose gels showing the DNA ladder formation, or DNA fragmentation, that occurs in apoptotic cells. Lane 2 shows control cells, lanes 3, 4 and 5 show UVC, anti-Fas and sorbitol treated cells. A 100-bp size marker is shown in Lane 1.

**Figure 2:** (A) IR absorption spectra in the 1800-800 cm⁻¹ region, for control *(black)*, and anti-Fas *(red)* treated A3 dried cell suspensions. All spectra were obtained from a single preparation. 23 and 26 spectra were collected for control and anti-Fas treated samples. Each spectrum shown results from the coaddition of 64 interferograms. All spectra are at 4 cm⁻¹ spectral resolution. (B) The same spectra as in (A) anti-Faser standardization. For standardization, the mean absorbance is subtracted from the spectrum, which is then divided by the standard deviation. (C) Average of the spectra in (B). The error bars represent the standard deviation (plus/minus) of all of the spectra shown in (B) at each frequency. (D) Averaged spectra shown in (C) without the error bars. (E) Same as the spectra in (D) except results are for five separate experiments for cells grown and treated on different days. Spectra on each day are shifted vertically for ease of comparison. Each spectrum in (E) is the average of ~25 spectra.
**Figure 3:** Standardized IR absorption spectra (*top*), second derivative spectra (*bottom*) and absorbance difference spectra (*middle*) in the (A) 1770-1450, (B) 1500-1150 and (C) 1150-900 cm\(^{-1}\) regions, for control A3 cells (*black*), control for A3 cells that underwent the same treatment as UVC irradiated cells (*red*), sorbitol treated A3 cells (*magenta*), anti-Fas treated A3 cells (*green*) and A3 cells that were irradiated with UVC light (*blue*). Absorbance spectra are the average of six independent measurements. In each experiment ~25 spectra were collected from different points on 2-3 sample “drops”. The second derivative spectra are calculated from the absorbance spectra and have not been smoothed. The second derivative spectra were scaled by 100 and shifted vertically for ease of comparison. Corresponding absorbance and second derivative spectra are displayed using the same color scheme. Five FTIR absorbance difference spectra were calculated from the absorbance spectra, and are numbered and colored differently according to the labels. For example, difference spectrum 1 is the subtraction of the spectrum of control cells from the spectrum of anti-Fas treated cells. All absorbance difference spectra have been scaled by a factor of three for ease of viewing. *Abbreviations:* AF - anti-Fas, S - sorbitol, UVT - UV treated, CUV - UV control, C-control.

**Figure 4:** (A) Inner (black) and intra (red) difference spectra calculated from spectra of control and anti-Fas-treated cells obtained in six different experiments. The individual spectra used to calculate the two averaged spectra in each experiment are chosen at random. Inner difference spectra: (control\(_1\) minus control\(_2\)) and (anti-Fas treated\(_1\) minus anti-Fas treated\(_2\)). Intra difference spectra: (control\(_1\) minus anti-Fas treated\(_1\)) and (control\(_2\) minus anti-Fas treated\(_2\)). (B) p-values calculated using the WRST applied to the intra difference spectra shown in Figure 4A. The inset shows an expanded view of the p-value data.
Figure 1
Figure 2
Figure 3
Figure 4
5.2 Supplementary information for analyzing T-Cells datasets

5.2.1 Principal component discriminant analysis

As described in section 5.1, FTIR raw T-Cell data measured in 6 different days were used in this analysis. Since pair-wise comparison can only work for two group comparisons, here we apply a multi-group comparison method, PCA-LDA, to explore and compare the different results. We categorized five different treatments into five different clusters, which are AntiFas (AF), Control (C), ControlUV (CUV or CU), Sorbitol (S) and UVtreated (UV), and treated them together as a whole.

The same procedures (PCA-LDA with LOOCV using SAS (SAS Institute Inc., Cary, NC, USA)) that were used for Vero cell analysis were applied to T-Cell data, as described in the previous chapter.

Data analysis procedures are listed below:

1. Data were standardized for each treatment separately. Standardization method is to standardize data through frequencies. In other words, if mean = mean of absorbance (Abs) of 365 frequencies (there are 365 frequencies total), and sd = second deviation of 300 frequency abs., then (abs-mean)/sd. All data were merged into one file using MATLAB and mark each day as 1 to 6 and marked for each treatment as 1 to 5 (AntiFas=1, Control=2, ControlUV=3, Sorbitol=4 and UVtreated=5).

2. Data structure were indicated as in Table 5-1 below: 365 variables (Var, wavenumbers), 721 observations (Obs) (~120 spectra for each treatment), 5 treatments (inf) : (af, c, cuv, s, uv), 6 days of repeats (date). Note: Treatment S data only has 5 days of repeats, as day 3 was missing here.
3. To analyze the dataset and distinguish each treatment, principal component discriminant analysis (PCA-LDA) was applied to the standardized dataset. All multivariate analysis was performed using SAS 9.1 (SAS Institute Inc., Cary, NC, USA). The merged file was processed first using the principal component discriminant analysis (PCA-LDA) method as well as the leave-one-out cross-validation.

4. Dataset was imported in SAS and then the principle components (PCs) were calculated. The first 25 principle components, representing nearly 95% of all the information from the spectra, are applied to the linear discriminant analysis (LDA).

5. How many PCs we chose depended on what level of discriminant level (sensitivity and specificity) we required. In order to find the best number of PCs we repeated the PCA-LDA analysis several times by increasing the number of PCs we used. The number of PCs was increased starting from 2 until the associated highest classification accuracies (sensitivity and specificity) were reached. For our dataset, 25 was a good number of PCs when balancing the minimum information input and better classification accuracies.

6. The classification accuracies, sensitivity and specificity, were computed by performing the leave-one-out cross validation method. Leave-one-out is the degenerate case of K-Fold Cross Validation, where K is chosen as the total number of total spectra. Leave-one-out cross-validation (LOOCV) involves using a single observation from the original data as the validation data, and the remaining observations as the training data. This is repeated such that each observation in the sample is used once as the validation data. Therefore, the averages of all sensitivity and specificity were the final classification accuracies.
To better visualize the separation among clusters produced by this Multivariate analysis, one 3D plot and one 2D plot (Figure 5-1) below were drawn by MatLab R2009a (The Mathworks Inc. Natick, MA), where each cluster corresponded to the differentiation treatment for T-Cells. AntiFas is in blue. Control is in red. ControlUV is in cyan. Sorbitol is in magenta. UVTeated is in green.

5.2.1.1 Results and Discussions

Analysis results are presented in Table 5-2 and Figure 5-1. In Figure 5-1, it can be seen from the 2D or 3D plot that the control is well-separated from the remaining groups and that each cluster only slightly overlaps with any nearby group. The 100% sensitivity and 99.8% specificity in Table 5-2 also indicate that the control is well-separated from the remaining groups. We also show good discrimination among all five clusters, which should indicate that PCA-LDA with LOOCV is a good method for multi-group analysis.

We also examined the results by using different numbers of PCs. Table 5-3 presents results for PCA-LDA with 365 PCs applied in the analysis with a nearly 100% sensitivity and specificity. As the number of PCs decrease, classification ability decreased dramatically as shown in Table 5-4 (10 PCs) and Table 5-5 (7 PCs). This result agrees with the hypothesis that the more information (more PCs) from the datasets we have, the better the discrimination ability that can be achieved.
Figure 5-1 Scatter Plots of PCA-LDA Using 25 PCs with 2D and 3D view.
Table 5-1 Data structure for all T-Cell with five different treatments.

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<tr>
<th>Abs1</th>
<th>Var1</th>
<th>...</th>
<th>Var365</th>
<th>inf</th>
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<td>6</td>
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<td></td>
</tr>
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<tr>
<td>Abs721</td>
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<td>6</td>
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Table 5-2 Results for PCA-LDA with LOOCV 25 PCs applied in the analysis.

<table>
<thead>
<tr>
<th>FT-IR prediction</th>
<th>AntiFas</th>
<th>Control</th>
<th>ControlUV</th>
<th>Sorbitol</th>
<th>UVTreated</th>
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<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>AntiFas</td>
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<td>0</td>
<td>3</td>
<td>14</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>148</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ControlUV</td>
<td>2</td>
<td>1</td>
<td>131</td>
<td>0</td>
<td>19</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>125</td>
<td>0</td>
</tr>
<tr>
<td>UVTreated</td>
<td>5</td>
<td>11</td>
<td>0</td>
<td>15</td>
<td>127</td>
</tr>
<tr>
<td>Number Correct</td>
<td>123</td>
<td>148</td>
<td>131</td>
<td>125</td>
<td>127</td>
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<table>
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<tr>
<th></th>
<th>Sensitivity</th>
<th>Specificity</th>
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<tr>
<td></td>
<td>87.86%</td>
<td>96.90%</td>
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<tr>
<td>AntiFas</td>
<td>100.00%</td>
<td>99.80%</td>
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<tr>
<td>Control</td>
<td>85.62%</td>
<td>97.21%</td>
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<td>ControlUV</td>
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<td>99.44%</td>
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<td>Sorbitol</td>
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<td>94.11%</td>
</tr>
<tr>
<td>UVTreated</td>
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</tr>
<tr>
<td>Number Correct</td>
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<td></td>
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### Table 5-3 Results for PCA-LDA with 365 PCs applied in the analysis.

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<th>Control</th>
<th>ControlUV</th>
<th>Sorbitol</th>
<th>UVTreated</th>
</tr>
</thead>
<tbody>
<tr>
<td>AntiFas</td>
<td>1</td>
<td>139</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>2</td>
<td>0</td>
<td>148</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ControlUV</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>150</td>
<td>1</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>123</td>
</tr>
<tr>
<td>UVTreated</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Number Correct</td>
<td>139</td>
<td>148</td>
<td>150</td>
<td>123</td>
<td>147</td>
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<table>
<thead>
<tr>
<th></th>
<th>sensitivity</th>
<th>specificity</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>99.29%</td>
<td>100.00%</td>
</tr>
<tr>
<td></td>
<td>99.34%</td>
<td>95.35%</td>
</tr>
<tr>
<td></td>
<td>96.08%</td>
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### Table 5-4 Results for PCA-LDA with 10 PCs applied in the analysis.

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<tr>
<td>AntiFas</td>
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<td>Control</td>
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<td>146</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>ControlUV</td>
<td>3</td>
<td>0</td>
<td>5</td>
<td>124</td>
<td>1</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>4</td>
<td>16</td>
<td>2</td>
<td>0</td>
<td>104</td>
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<td>UVTreated</td>
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<td>32</td>
<td>0</td>
<td>24</td>
<td>18</td>
</tr>
<tr>
<td>Number Correct</td>
<td>85</td>
<td>146</td>
<td>124</td>
<td>104</td>
<td>79</td>
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</table>

<table>
<thead>
<tr>
<th></th>
<th>sensitivity</th>
<th>specificity</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>60.71%</td>
<td>98.65%</td>
</tr>
<tr>
<td></td>
<td>82.12%</td>
<td>80.62%</td>
</tr>
<tr>
<td></td>
<td>51.63%</td>
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sensitivity
specificity
Table 5-5 Results for PCA-LDA with 7 PCs applied in the analysis.

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<th>FT-IR prediction</th>
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<th>Sorbitol</th>
<th>UVTreated</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>AntiFas</td>
<td>84</td>
<td>2</td>
<td>0</td>
<td>23</td>
<td>31</td>
</tr>
<tr>
<td>Control</td>
<td>2</td>
<td>123</td>
<td>15</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>ControlUV</td>
<td>3</td>
<td>22</td>
<td>108</td>
<td>5</td>
<td>16</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>4</td>
<td>16</td>
<td>6</td>
<td>1</td>
<td>98</td>
</tr>
<tr>
<td>UVTreated</td>
<td>5</td>
<td>44</td>
<td>0</td>
<td>25</td>
<td>12</td>
</tr>
<tr>
<td>Number Correct</td>
<td>84</td>
<td>123</td>
<td>108</td>
<td>98</td>
<td>72</td>
</tr>
</tbody>
</table>

| sensitivity       | 60.00%  | 83.11%  | 71.52%    | 75.97%   | 47.06%    |
| specificity       | 86.80%  | 92.35%  | 90.19%    | 88.76%   | 88.25%    |

We have performed PCA-LDA with leave-one-out cross validation. The results show the same level of accuracy of discrimination as described in the literature [30]. However, we encountered another problem due to the same variation of the spectra we collected from different days.

Leave-one-out cross-validation (LOOCV) involves using a single spectrum from all the spectra collected over different days as the validation data, and the remaining spectra as the training data. This is the same as a K-fold cross-validation with K being equal to the number of observations in the original sample. If the data collected over different days proves to be consistent (no statistical difference over days among all spectra) or the distribution of the observed sample is the same as the population distribution, we will get similar results from K-fold cross-validation (with k equal to any number) as the results we got from leave-one-out, which represents a fair cross validation.

However, we obtained a much lower sensitivity and specificity from leaving one out of six days (all the spectra collected from one day) out of the cross validation (6-fold CV) (refer
to 5.2.2), which showed that our sample size is too small to apply the PCA-LDA k-fold CV method. Statistically speaking, 20 independent datasets will be a good sample size for any type of statistical analysis. Therefore, we have to employ a new method to reduce the influence of the variation from the different days due to our current sample size. Thus, we have settled on the new pair wise comparison method (as we discussed in 5.1) that we applied to our T-Cell datasets with a sample size equal to 6.

In the process of pair wise comparison, the most important step is to use inner intra differences. Naturally, one question will arise: why do we use inner intra differences? In order to test whether there is a difference between population means of any two groups (two different treatments), our data should satisfy two conditions:

1. The two populations have the same variance. This assumption is called the assumption of homogeneity of variance. To prove this assumption, we have to prove that the variances of inner-differences within treatment type 1 should be the same as that within treatment type 2. We applied a homogeneous variance test to the two kinds of inner difference, and the homogeneous variance was confirmed.

2. The populations are normally distributed. We randomly divided all spectra observation points into two equal groups for each treatment, and find their average absorbances and inner and intra differences. From central limit theorem, both inner and intra-differences have approximately normal distributions.

Only by satisfying these two conditions, can the statistical results derived from all the statistical methods be treated as reliable. However, most of the statistical methods employed in the
literature do not attempt to meet these conditions; therefore, it is unclear whether or not the results from those studies are statistically significant [30].

In order to reduce the influence of the variability among the data observed in different dates, we will only use the differences of the absorbances between two groups (two different treatments) from the same date. To apply pair wise comparison, a control group was created by finding the difference between two spectra from treatment 1 and two spectra from treatment 2. Before the subtraction, we randomly divided all spectral observation points into two equal groups for two different treatments, and found their average absorbances. The differences between the same treatments are called inner-difference, and the differences between different treatments are called intra-difference.

5.2.2 Linear regression analysis

As we discussed above, although the results from the PCA-LDA with LOOCV method turns out to indicate a good discrimination, the first LOOCV validation method does not prove to be sufficient to predict the new data due to the large variation from our dataset of each day. In order to improve the PCA-LDA method, we expanded our test data size and used each single day for all five treatments as the test dataset and the other 5 days as the model dataset, which is the same as 6-fold cross validation. All the remaining parts of the method remain the same as in section 5.2.

To find the most significant variables, dummy variable coding was applied for linear regression on each wavenumber, repeating the same procedure for all 365 wavenumbers. Each regression returns one result with the p-value score. Only 37 variables, with a P-value smaller than 0.0002, were then selected for principle component calculation. Based on all 37 variables, the
first 25 PCs were calculated and input into LDA. To validate the model, leave one-day–out cross validation was applied to calculate the sensitivity and specificity for each treatment. Averaged sensitivity and specificity are listed in the table below.

To find out the shrinkage and the final sensitivity:

1. Pick out the variables with P-value < 0.0002 from model in stand of from all 5 single values (intercept, af, cuv, s, uv)

2. Apply regression method (REG) to all 6 day’s datasets. 345 variables were found out to be significant. Find out sensitivity for each type, called model sensitivity.

   (No specificity needed here due to a multi-group comparison).

3. Apply REG for five of the 6 days data. 339 variables were found to be significant.

   Find out the sensitivity for each type, called model sensitivity.

4. Use the same variables from model1 - 339 variables - to test left-out dataset.

   Find out the sensitivity for each type, called test1 sensitivity.

5. Steps 2 and 3 are repeated for all 6 days datasets.

   Find the average of model1-model6 sensitivity, and the average of the test1-test6 sensitivity.

6. Find the shrinkage using average of model1-6 sensitivity minus average of test1-6 sensitivity.

7. Find the final sensitivity using model sensitivity from step 1 minus the shrinkage.
5.2.2.1 Results

Table 5-7 is an output from PCA-LDA method with leave-one-day-out cross validation (left) and leave-two-days-out cross validation (right). Table 5-8 displays the results from applying regression analysis with the PCA-LDA method, along with validation using leave-one-day-out cross validation (left) and leave-two-days-out cross validation (right).

The results below did not satisfy the classification requirement (less than 50%). However, comparing the left side of Table 5-6 and 5-7, there is a small improvement on the shrinkage due to the regression analysis, and final sensitivity has been slightly increased by 0.1% for table 5-7, which indicates that regression analysis is a good approach for picking out the significant variables. Cross validation with leaving two days out produces smaller sensitivity rather than from leaving one day out. Again, this indicates that our datasets have substantial day-to-day variation.
Table 5-6 Results for only apply PCA-LDA (No regression analysis applied) with 25 PCs.

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<th></th>
<th>Leave One Day Out</th>
<th>Leave Two Days Out</th>
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<tbody>
<tr>
<td></td>
<td>Model sensitivity</td>
<td>shrinkage</td>
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<tr>
<td>Sensitivity for AF</td>
<td>0.921429</td>
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<tr>
<td>Sensitivity for C</td>
<td>1</td>
<td>0.624676</td>
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<tr>
<td>Sensitivity for CUV</td>
<td>0.854305</td>
<td>0.334422</td>
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<td>Sensitivity for S</td>
<td>0.790698</td>
<td>0.702369</td>
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<tr>
<td>Sensitivity for UV</td>
<td>0.777778</td>
<td>0.453766</td>
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</tbody>
</table>

Table 5-7 Results for applying regression analysis and PCA-LDA by using dummy coding REG with 25 PCs.

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<th>Leave One Day Out</th>
<th>Leave Two Days Out</th>
</tr>
</thead>
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<td></td>
<td>Model sensitivity</td>
<td>shrinkage</td>
</tr>
<tr>
<td>Sensitivity for AF</td>
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<td>0.297309</td>
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<tr>
<td>Sensitivity for C</td>
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<td>0.509837</td>
</tr>
<tr>
<td>Sensitivity for CUV</td>
<td>0.854305</td>
<td>0.56418</td>
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<tr>
<td>Sensitivity for S</td>
<td>0.821705</td>
<td>0.521282</td>
</tr>
<tr>
<td>Sensitivity for UV</td>
<td>0.751634</td>
<td>0.580397</td>
</tr>
</tbody>
</table>

5.2.3 “Summing by five” procedure

Before we applied pair wise comparison, we performed the sum-by-five procedure.

Summing by five is a routine procedure for minimizing the data variation caused by slight wave-number shifting, which we first developed for comparing mock and HSV1 infected data sets.

Since we obtained great accuracies from the method we developed, we naturally applied the same procedure to the T-Cell data. However, it was necessary to determine that summing by five
was a helpful step for analyzing the T-Cell data. Therefore, we applied the same method with and without summing by five.

Each comparison group (AF vs C, AF vs CUV, AF vs UV, C vs S, CUV vs S, S vs UV) has two figures (inner intra difference data plot and P-value plot) for data processed with or without summing by five and one result table for the data processed with and without summing by five together. Figure 5-2 plots the intra (red) and inner (blue) differences for AF vs C. Figure 5-3 shows the p-value plot for the AF vs C with and without summing-by-five. Similarly, Figure 5-4 plots the intra (red) and inner (blue) differences for AF vs CUV. Figure 5-5 shows the p-value plot for the AF vs CUV with and without summing-by-five, so on through S vs UV.

Table 5-8 shows results for AF vs C from multivariate statistical analysis with the summing-by-five step (Table A) and without summing-by-five (Table B). Similarly, Table 5-9 shows results for AF vs CUV from multivariate statistical analysis with the summing-by-five step (Table A) and without summing-by-five (Table B), so on through S vs. UV. For each table, we employed two methods to determine the model and test datasets. First, we applied 2-fold cross validation, which normally gives an under-estimated shrinkage. The intra or inner differences were split into model and test groups, called model intra and test intra or model inner and test inner. We applied WSRT only on the model intra data to determine the significant frequencies. PLSR was then applied to the selected frequencies for model intra and model inner data sets. By applying the same discriminators for PLSR as for the model intra and inner sets, we were able to calculate the predicted values for the test sets. We calculated sensitivity, specificity and AUC from the predicted values of PLSR for both model and test sets. By finding the difference between these two specificities, we obtained estimates of the shrinkage (Col3). Specificity of Cross Vali-
dation (Col4) was calculated by subtracting this shrinkage (Col3) from the specificity for original data (Col2).

The second method we used to determine the model and test datasets was the bootstrap method, which normally gives an over-estimated shrinkage. Model datasets for bootstrap were calculated by constructing a number of resamples. Resampling entails drawing randomly with replacement from a set of data points of the observed dataset, which are of equal size to the original observed datasets. Test datasets refers to the original observed dataset. By applying WSRT only on the model intra data, we determined the significant frequencies. PLSR was then applied to the selected frequencies for model intra and inner data. By applying the same discriminators for PLSR as for model datasets, predicted values of PLSR were calculated for the test datasets. Following the same procedures as for CV, we calculated column 5, shrinkage from Bootstrap, and Column 6, Specificity calculated using Bootstrap. Col 6 was obtained by subtracting column 5 from column 1. By finding the average of these two specificities, as in column 7, we can have fairly accurate final specificities and AUC for three different sensitivities.

All the results indicated that not using the “summing-by-five” step seems to increase the values for specificities and AUC, except for the comparison for AF vs CUV. As can be seen in p-value plot, summing by five not only smooth the data but also overwrites the fine details of the spectra, thereby, decreasing the information from spectra as well as the discrimination ability. Again, it proves that the discrimination method is highly depended upon the dataset itself.
Figure 5-2 Plot of intra (red) and inner (blue) differences for AF vs C.
With summing by 5:

![P-value plot 1]

Without summing by 5:

![P-value plot 2]

Figure 5-3 P-value plot for AF vs C.
Table 5-8 Results for AF vs C from multivariate statistical analysis with the summing-by-five step (Table A) and without summing by 5 (Table B).

<table>
<thead>
<tr>
<th>Column 1</th>
<th>Column 2</th>
<th>Column 4</th>
<th>Column 3</th>
<th>Column 6</th>
<th>Column 5</th>
<th>Column 7</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Table A</strong></td>
<td>Specificity for original data</td>
<td>Specificity of cross validation(CV)</td>
<td>Shrinkage from CV</td>
<td>Specificity of boots trap(BT)</td>
<td>Shrinkage from BT</td>
<td>Average of Specificity between CV and BT</td>
</tr>
<tr>
<td>Sensitivity=95%</td>
<td>1</td>
<td>0.417231</td>
<td>0.582769</td>
<td>0.900246</td>
<td>0.099754</td>
<td>0.658739</td>
</tr>
<tr>
<td>Sensitivity=90%</td>
<td>1</td>
<td>0.575665</td>
<td>0.424335</td>
<td>0.947589</td>
<td>0.052411</td>
<td>0.761627</td>
</tr>
<tr>
<td>Sensitivity=80%</td>
<td>1</td>
<td>0.770638</td>
<td>0.229362</td>
<td>0.987636</td>
<td>0.012364</td>
<td>0.879137</td>
</tr>
<tr>
<td><strong>AUC</strong></td>
<td>1</td>
<td>0.868054</td>
<td>0.131946</td>
<td>0.983807</td>
<td>0.016193</td>
<td>0.92593</td>
</tr>
</tbody>
</table>

| **Table B** | Specificity for original data | Specificity of cross validation(CV) | Shrinkage from CV | Specificity of boots trap(BT) | Shrinkage from BT | Average of Specificity between CV and BT |
| Sensitivity=95% | 1 | 0.468454 | 0.531546 | 0.897549 | 0.102451 | 0.683002 |
| Sensitivity=90% | 1 | 0.62774 | 0.37226 | 0.962876 | 0.037124 | 0.795308 |
| Sensitivity=80% | 1 | 0.814154 | 0.185846 | 0.994723 | 0.005277 | 0.904438 |
| **AUC** | 1 | 0.895182 | 0.104818 | 0.985263 | 0.014737 | 0.940222 |
Figure 5-4 Plot of intra (red) and inner (blue) differences for AF vs CUV.
With summing by 5:

$P$-value

Figure 5-5 P-value plot for AF vs CUV.

Without summing by 5:

$P$-value

Figure 5-5 P-value plot for AF vs CUV.
Table 5-9 Results for AF vs CUV from multivariate statistical analysis with the summing-by-five step (Table A) and without summing by 5 (Table B).

<table>
<thead>
<tr>
<th>Column 1</th>
<th>Column 2</th>
<th>Column 4</th>
<th>Column 3</th>
<th>Column 5</th>
<th>Column 6</th>
<th>Column 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table A</td>
<td>Specificity for original data</td>
<td>Specificity of cross validation (CV)</td>
<td>Shrinkage from CV</td>
<td>Specificity of boots trap (BT)</td>
<td>Shrinkage from BT</td>
<td>Average of Specificity between CV and BT</td>
</tr>
<tr>
<td>Sensitivity=95%</td>
<td>1</td>
<td>0.558799</td>
<td>0.441201</td>
<td>0.736558</td>
<td>0.263442</td>
<td>0.647678</td>
</tr>
<tr>
<td>Sensitivity=90%</td>
<td>1</td>
<td>0.66786</td>
<td>0.33214</td>
<td>0.823657</td>
<td>0.176343</td>
<td>0.745759</td>
</tr>
<tr>
<td>Sensitivity=80%</td>
<td>1</td>
<td>0.748701</td>
<td>0.251299</td>
<td>0.944113</td>
<td>0.055877</td>
<td>0.846407</td>
</tr>
<tr>
<td>AUC</td>
<td>1</td>
<td>0.876465</td>
<td>0.123535</td>
<td>0.954322</td>
<td>0.045678</td>
<td>0.915393</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table B</th>
<th>Specificity for original data</th>
<th>Specificity of cross validation (CV)</th>
<th>Shrinkage from CV</th>
<th>Specificity of boots trap (BT)</th>
<th>Shrinkage from BT</th>
<th>Average of Specificity between CV and BT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity=95%</td>
<td>1</td>
<td>0.533018</td>
<td>0.466982</td>
<td>0.724671</td>
<td>0.275329</td>
<td>0.628845</td>
</tr>
<tr>
<td>Sensitivity=90%</td>
<td>1</td>
<td>0.660364</td>
<td>0.339636</td>
<td>0.812766</td>
<td>0.187234</td>
<td>0.736565</td>
</tr>
<tr>
<td>Sensitivity=80%</td>
<td>1</td>
<td>0.725095</td>
<td>0.274905</td>
<td>0.94302</td>
<td>0.05698</td>
<td>0.834057</td>
</tr>
<tr>
<td>AUC</td>
<td>1</td>
<td>0.858824</td>
<td>0.141176</td>
<td>0.954524</td>
<td>0.045476</td>
<td>0.906674</td>
</tr>
</tbody>
</table>
Figure 5-6 Plot of intra (red) and inner (blue) differences for AF vs UV.
With summing by 5:

![P-value plot 1](image1)

Without summing by 5:

![P-value plot 2](image2)

Figure 5-7 P-value plot for AF vs UV.
Table 5-10 Results for AF vs UV from multivariate statistical analysis with the summing-by-five step (Table A) and without summing by 5 (Table B).

<table>
<thead>
<tr>
<th>Column 1</th>
<th>Column 2</th>
<th>Column 4</th>
<th>Column 3</th>
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<th>Column 5</th>
<th>Column 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table A</td>
<td>Specificity for original data</td>
<td>Specificity of cross validation(CV)</td>
<td>Shrinkage from CV</td>
<td>Specificity of boots trap(BT)</td>
<td>Shrinkage from BT</td>
<td>Average of Specificity between CV and BT</td>
</tr>
<tr>
<td>Sensitivity=95%</td>
<td>1</td>
<td>0.126495</td>
<td>0.873505</td>
<td>0.633311</td>
<td>0.366689</td>
<td>0.379903</td>
</tr>
<tr>
<td>Sensitivity=90%</td>
<td>1</td>
<td>0.198035</td>
<td>0.801965</td>
<td>0.741243</td>
<td>0.258757</td>
<td>0.469639</td>
</tr>
<tr>
<td>Sensitivity=80%</td>
<td>1</td>
<td>0.40181</td>
<td>0.59819</td>
<td>0.877079</td>
<td>0.122921</td>
<td>0.639444</td>
</tr>
<tr>
<td>AUC</td>
<td>1</td>
<td>0.659719</td>
<td>0.340281</td>
<td>0.926099</td>
<td>0.073901</td>
<td>0.792909</td>
</tr>
<tr>
<td>Table B</td>
<td>Specificity for original data</td>
<td>Specificity of cross validation(CV)</td>
<td>Shrinkage from CV</td>
<td>Specificity of boots trap(BT)</td>
<td>Shrinkage from BT</td>
<td>Average of Specificity between CV and BT</td>
</tr>
<tr>
<td>Sensitivity=95%</td>
<td>1</td>
<td>0.155769</td>
<td>0.844231</td>
<td>0.716519</td>
<td>0.283481</td>
<td>0.436144</td>
</tr>
<tr>
<td>Sensitivity=90%</td>
<td>1</td>
<td>0.280485</td>
<td>0.719515</td>
<td>0.829327</td>
<td>0.170673</td>
<td>0.554906</td>
</tr>
<tr>
<td>Sensitivity=80%</td>
<td>1</td>
<td>0.521599</td>
<td>0.478401</td>
<td>0.949026</td>
<td>0.050974</td>
<td>0.735313</td>
</tr>
<tr>
<td>AUC</td>
<td>1</td>
<td>0.727681</td>
<td>0.272319</td>
<td>0.955183</td>
<td>0.044817</td>
<td>0.841432</td>
</tr>
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</table>
Figure 5-8 Plot of intra (red) and inner (blue) differences for C vs S.
With summing by 5:

![P-value plot with summing by 5]

Without summing by 5:

![P-value plot without summing by 5]

Figure 5-9 P-value plot for C vs S.
Table 5-11 Results for C vs S from multivariate statistical analysis with the summing-by-five step (Table A) and without summing by 5 (Table B).

<table>
<thead>
<tr>
<th>Column 1</th>
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<tr>
<td><strong>Table A</strong></td>
<td>Specificity for original data</td>
<td>Specificity of cross validation(CV)</td>
<td>Shrinkage from CV</td>
<td>Specificity of boots trap(BT)</td>
<td>Shrinkage from BT</td>
<td>Average of Specificity between CV and BT</td>
</tr>
<tr>
<td>Sensitivity=95%</td>
<td>1</td>
<td>0.192917</td>
<td>0.807083</td>
<td>0.883527</td>
<td>0.116473</td>
<td>0.538222</td>
</tr>
<tr>
<td>Sensitivity=90%</td>
<td>1</td>
<td>0.28253</td>
<td>0.71747</td>
<td>0.937295</td>
<td>0.062705</td>
<td>0.609912</td>
</tr>
<tr>
<td>Sensitivity=80%</td>
<td>1</td>
<td>0.438371</td>
<td>0.561629</td>
<td>0.986974</td>
<td>0.013026</td>
<td>0.712672</td>
</tr>
<tr>
<td>AUC</td>
<td>1</td>
<td>0.700088</td>
<td>0.299912</td>
<td>0.983701</td>
<td>0.016299</td>
<td>0.841894</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Column 1</th>
<th>Column 2</th>
<th>Column 4</th>
<th>Column 3</th>
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<th>Column 5</th>
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</tr>
</thead>
<tbody>
<tr>
<td><strong>Table B</strong></td>
<td>Specificity for original data</td>
<td>Specificity of cross validation(CV)</td>
<td>Shrinkage from CV</td>
<td>Specificity of boots trap(BT)</td>
<td>Shrinkage from BT</td>
<td>Average of Specificity between CV and BT</td>
</tr>
<tr>
<td>Sensitivity=95%</td>
<td>1</td>
<td>0.279215</td>
<td>0.720785</td>
<td>0.836632</td>
<td>0.163368</td>
<td>0.557923</td>
</tr>
<tr>
<td>Sensitivity=90%</td>
<td>1</td>
<td>0.324684</td>
<td>0.675316</td>
<td>0.92501</td>
<td>0.07499</td>
<td>0.624847</td>
</tr>
<tr>
<td>Sensitivity=80%</td>
<td>1</td>
<td>0.443004</td>
<td>0.556996</td>
<td>0.985951</td>
<td>0.014049</td>
<td>0.714478</td>
</tr>
<tr>
<td>AUC</td>
<td>1</td>
<td>0.70921</td>
<td>0.29079</td>
<td>0.978099</td>
<td>0.021901</td>
<td>0.843655</td>
</tr>
</tbody>
</table>
Figure 5-10 Plot of intra (red) and inner (blue) differences for CUV vs S.
With summing by 5:

\[ P-value \]

\[ \begin{array}{c}
\text{pValue} \\
1.0000 \\
0.9000 \\
0.8000 \\
0.7000 \\
0.6000 \\
0.5000 \\
0.4000 \\
0.3000 \\
0.2000 \\
0.1000 \\
0.0000
\end{array} \]

\[ x \]

\[ \begin{array}{c}
700 \\
800 \\
900 \\
1000 \\
1100 \\
1200 \\
1300 \\
1400 \\
1500 \\
1600
\end{array} \]

\text{blue—inner difference, red— intra difference}

Without summing by 5:

\[ P-value \]

\[ \begin{array}{c}
\text{pValue} \\
1.0000 \\
0.9000 \\
0.8000 \\
0.7000 \\
0.6000 \\
0.5000 \\
0.4000 \\
0.3000 \\
0.2000 \\
0.1000 \\
0.0000
\end{array} \]

\[ x \]

\[ \begin{array}{c}
700 \\
800 \\
900 \\
1000 \\
1100 \\
1200 \\
1300 \\
1400 \\
1500 \\
1600
\end{array} \]

\text{blue—inner difference, red— intra difference}

Figure 5-11 P-value plot for CUV vs. S.
Table 5-12 Results for CUV vs S from multivariate statistical analysis with the summing-by-five step (Table A) and without summing by 5 (Table B).

<table>
<thead>
<tr>
<th>Column 1</th>
<th>Column 2</th>
<th>Column 4</th>
<th>Column 3</th>
<th>Column 6</th>
<th>Column 5</th>
<th>Column 7</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Table A</strong></td>
<td>Specificity for original data</td>
<td>Specificity of cross validation (CV)</td>
<td>Shrinkage from CV</td>
<td>Specificity of boots trap (BT)</td>
<td>Shrinkage from BT</td>
<td>Average of Specificity between CV and BT</td>
</tr>
<tr>
<td>Sensitivity=95%</td>
<td>1</td>
<td>0.483349</td>
<td>0.516651</td>
<td>0.765434</td>
<td>0.07929</td>
<td>0.624391</td>
</tr>
<tr>
<td>Sensitivity=90%</td>
<td>1</td>
<td>0.566802</td>
<td>0.433198</td>
<td>0.861608</td>
<td>0.05832 7</td>
<td>0.714205</td>
</tr>
<tr>
<td>Sensitivity=80%</td>
<td>1</td>
<td>0.640094</td>
<td>0.359906</td>
<td>0.924422</td>
<td>0.04863 6</td>
<td>0.782258</td>
</tr>
<tr>
<td>AUC</td>
<td>1</td>
<td>0.795005</td>
<td>0.204995</td>
<td>0.952125</td>
<td>0.02240 5</td>
<td>0.873565</td>
</tr>
</tbody>
</table>

| **Table B** | Specificity for original data | Specificity of cross validation (CV) | Shrinkage from CV | Specificity of boots trap (BT) | Shrinkage from BT | Average of Specificity between CV and BT |
| Sensitivity=95% | 1 | 0.360015 | 0.639985 | 0.850202 | 0.14979 7 | 0.605108 |
| Sensitivity=90% | 1 | 0.447298 | 0.552702 | 0.893942 | 0.10605 8 | 0.67062 |
| Sensitivity=80% | 1 | 0.63202 | 0.36798 | 0.929031 | 0.07096 9 | 0.780525 |
| AUC | 1 | 0.79129 | 0.20871 | 0.963318 | 0.03668 | 0.877304 |
Figure 5-12 Plot of intra (red) and inner (blue) differences for UV vs S.
With summing by 5

![P-value plot for UV vs. S](image1)

Without summing by 5:

![P-value plot for UV vs. S](image2)

Figure 5-13 P-value plot for UV vs. S
Table 5-13 Results for S vs. UV from multivariate statistical analysis with the summing-by-five step (Table A) and without summing by 5 (Table B).

<table>
<thead>
<tr>
<th>Column 1</th>
<th>Column 2</th>
<th>Column 4</th>
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<th>Column 6</th>
<th>Column 5</th>
<th>Column 7</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Specificity for original data</td>
<td>Specificity of cross validation (CV)</td>
<td>Shrinkage from CV</td>
<td>Specificity of boots trap (BT)</td>
<td>Shrinkage from BT</td>
<td>Average of Specificity between CV and BT</td>
</tr>
<tr>
<td>Sensitivity=95%</td>
<td>1</td>
<td>0.141767</td>
<td>0.858233</td>
<td>0.679904</td>
<td>0.27175</td>
<td>0.410836</td>
</tr>
<tr>
<td>Sensitivity=90%</td>
<td>1</td>
<td>0.234523</td>
<td>0.765477</td>
<td>0.764398</td>
<td>0.19833</td>
<td>0.49946</td>
</tr>
<tr>
<td>Sensitivity=80%</td>
<td>1</td>
<td>0.376591</td>
<td>0.623409</td>
<td>0.846083</td>
<td>0.12957</td>
<td>0.611337</td>
</tr>
<tr>
<td>AUC</td>
<td>1</td>
<td>0.675637</td>
<td>0.324363</td>
<td>0.902798</td>
<td>0.08348</td>
<td>0.789217</td>
</tr>
</tbody>
</table>

| **Table B** | Specificity for original data | Specificity of cross validation(CV) | Shrinkage from CV | Specificity of boots trap(BT) | Shrinkage from BT | Average of Specificity between CV and BT |
| Sensitivity=95% | 1 | 0.124267 | 0.875733 | 0.686234 | 0.25856 | 0.405251 |
| Sensitivity=90% | 1 | 0.227962 | 0.772038 | 0.778943 | 0.17808 | 0.503452 |
| Sensitivity=80% | 1 | 0.404942 | 0.595058 | 0.878393 | 0.09642 | 0.641667 |
| AUC | 1 | 0.656552 | 0.343448 | 0.926709 | 0.06051 | 0.791631 |
5.2.4 A3 cell infected with HSV1

We also used A3 T-Cells infected with HSV1 in different MOI (0, 1, 2.5 and 5). The same pair-wise comparison procedure was performed on datasets infected with MOI=0 (mock) and MOI=1, 2.5 or 5, as described previously. As indicated in Table 5-14, we repeated experiments five times for MOI=1, nine times for MOI=2.5 and four times for MOI=5. Figure 5-14 shows the “Inner vs Intra” (Left) and “P-value” (Right) graphs, for comparison between uninfected T-Cell (mock) and T-Cell infected with HSV1 at MOI=1 (Top), 2.5 (Middle) and 5 (Bottom). It should be noted that the P-value plot for MOI=2.5 is dramatically different than the results from the other two. Fewer wavenumbers were selected as the significant variables under P-value equals to 0.05 at MOI=2.5.

Table 5-15 shows the results (specificities, shrinkage and AUC) from pair-wise comparison for T-Cells infected with HSV1 at different MOIs. In Table 5-15, results under the final specificity column indicate that MOI=5 has a better discriminating ability between infected and uninfected samples over MOI=2.5 and 1.

Table 5-16 shows the common wavenumbers for discriminating the HSV1 infected samples from the mock. Significant wavenumbers were selected from WSRT under P-value=0.05. The blue highlights the common wavenumbers for any two datasets with different MOI. The red highlights the common significant wavenumbers for all three datasets with MOI=1, 2.5 and 5. There are only six common wavenumbers selected for all three comparison groups: 1145, 1155, 1165, 1174, 1184, and 1550 cm$^{-1}$. Another feature revealed in Table 5-16 is that for MOI=1 and 5, P-values are much lower than those for MOI=2.5, which is also shown in the P-value plot for MOI=2.5 in Figure 5-14.
Figure 5-14 “Inner vs Intra” difference spectra (Left) and “P-value” (Right) graphs
Table 5-14 Experiment list for T-Cell infected with HSV1 at different MOI=0 (T-Cell mock), 1, 2.5 and 5.

<table>
<thead>
<tr>
<th>Date</th>
<th>T-Cell mock</th>
<th>T-Cell infected with HSV1 at MOI=1</th>
<th>T-Cell infected with HSV1 at MOI=2.5</th>
<th>T-Cell infected with HSV1 at MOI=5</th>
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</thead>
<tbody>
<tr>
<td>Day1</td>
<td>m, m</td>
<td>1, 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day2</td>
<td>M</td>
<td>1</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Day3</td>
<td>M</td>
<td>1, 1</td>
<td>5, 5</td>
<td></td>
</tr>
<tr>
<td>Day4</td>
<td>M</td>
<td></td>
<td>5</td>
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</tr>
<tr>
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<td>2.5, 2.5, 2.5</td>
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<td></td>
<td>2.5, 2.5, 2.5</td>
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<td>2.5, 2.5, 2.5</td>
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Table 5-15 Results from pair-wise comparison method for T-Cell data infected with different MOI.

<table>
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<tr>
<th>MOI=5 P-value(CV)=0.2 P-value(BT)=0.01</th>
<th>Specificity for CV model</th>
<th>Specificity for cross validation</th>
<th>Shrinkage for CV</th>
<th>Specificity for BT model</th>
<th>Specificity for BT test</th>
<th>Shrinkage for BT</th>
<th>Final shrinkage</th>
<th>Final specificity</th>
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<td>Sensitivity=95%</td>
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<td>0.46583</td>
<td>0.5341</td>
<td>1</td>
<td>0.90831</td>
<td>0.0916</td>
<td>0.3129</td>
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<tr>
<td>Sensitivity=90%</td>
<td>1</td>
<td>0.73993</td>
<td>0.2600</td>
<td>1</td>
<td>0.97393</td>
<td>0.0260</td>
<td>0.1430</td>
<td>0.8569</td>
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<tr>
<td>Sensitivity=80%</td>
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<td>0.93166</td>
<td>0.0683</td>
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<td>0.99960</td>
<td>0.0003</td>
<td>0.0343</td>
<td>0.9656</td>
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<td>AUC</td>
<td>1</td>
<td>0.92435</td>
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<td>1</td>
<td>0.98727</td>
<td>0.0127</td>
<td>0.0441</td>
<td>0.9558</td>
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<table>
<thead>
<tr>
<th>MOI=2.5 P-value(CV)=0.3 P-value(BT)=0.05</th>
<th>Specificity for CV model</th>
<th>Specificity for cross validation</th>
<th>Shrinkage for CV</th>
<th>Specificity for BT model</th>
<th>Specificity for BT test</th>
<th>Shrinkage for BT</th>
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<th>Final specificity</th>
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<td>0.12269</td>
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<td>0.96844</td>
<td>0.74950</td>
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<table>
<thead>
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<th>P-value(CV)=0.2</th>
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</tr>
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Table 5-16 Significant wavenumbers selected from WSRT under P-value=0.05.

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<td>P-value&lt;0.05</td>
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6 CONCLUSIONS

As we discussed in chapters two through four, infrared spectra obtained for Vero cells that are uninfected or infected with Herpes Simplex Virus type I can be distinguished using advanced statistical methods (pair-wise comparison) under certain experimental conditions.

From the results of T-cell analysis, with knowledge of apoptosis-induced spectral biomarkers we are able to (rapidly) assess how malignant cells respond (via apoptosis) to three different stresses. Thus a FTIR detection technology, together with knowledge of spectral biomarkers of malignant lymphocytes, may be a useful tool for rapidly testing drug resistance in leukemic cells directly isolated from patients.

The results of this study showed that Fourier transform infrared (FTIR) microscopy can be a promising analytical tool for detecting changes in cells due to viral infection and different treatment approaches under certain conditions. We can discriminate samples infected with viruses (HSV1 or coxsackie virus) from samples without infection at 6, 8, 12 and 24 hours post exposure. Different statistical analyses (PCA-LDA, LR and PLSR) and appropriate validation procedures were applied to our datasets. It should be noted that inner and intra variability should be carefully considered. We observed significant differences in specific areas of the spectra, which allowed us to obtain different levels of discriminations, and nearly perfect discriminations between samples with and without infection for pair wise comparison at certain hours post exposure.

For statistical analysis procedures, FTIR studies of cells in different states should include an assessment of variation within each treatment for each experiment and variation between different repeats. The methods used here to calculate sensitivity and specificity for distinguishing
spectra from cells under different treatments accurately reflect how well FTIR spectra from unknown cell types can be classified.

In summary, FTIR microscopy can be a useful analytical tool for detecting changes in cells under certain types of conditions, and it certainly seems worthwhile to continue with the development of FTIR microscopy for the purpose of viral infection diagnosis and treatment discrimination.
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