CHARACTERIZATION of NORMAL and MALIGNANT CELLS in CULTURE and HUMAN COLONIC TISSUES USING FTIR MICROSCOPIC SPECTROSCOPY, and ADVANCED COMPUTATIONAL METHODS

Thesis submitted in partial fulfillment of the requirements for the degree of

“DOCTOR OF PHILOSOPHY”

by

Ahmad Salman

Submitted to the Senate of Ben-Gurion University of the Negev

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Beer-Sheva
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Approved by the advisor: Prof. S. Mordechai

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Beer-Sheva
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1) Abstract

Apart from enormous efforts put in understanding, prevention and treatment, cancer remains a leading cause of death in developed countries. In addition to the obvious personal cost, cancer represents a significant economic burden to the state through both direct (diagnosis, therapeutics surgery etc.) and indirect (long term disability payments, taxation, revenue lose due to premature death etc.) costs. Perhaps, the most effective way to reduce both the personal and economic burden associated with cancer is early, accurate detection of the disease. Accurate early diagnosis potentially allows intervention when the disease is least aggressive, improving the chances of survival.

The current methods of diagnosis of cancer include a) tissue contrast b) physical examination c) MRI. Microscopic examination of sections of tissue requires processing of tissues (fixation, sectioning and staining) prior to analysis and it is prone to errors due to its inherent problems in differentiating pre-malignant and malignant cells with high accuracy. The physical examination of patients is inherently subjective and relies upon the experience of the physician. MRI, the youngest of these techniques is too expensive to be used for routine screening purpose.

Infrared (IR) spectroscopy is well known for its uniqueness as a noninvasive method in identifying vibrational structure of various materials. Huge quantity of literature is available on the structure and conformational aspects of proteins, nucleic acids and other biomolecules using infrared spectroscopy.

The IR wavelength, which is absorbed by the sample depends upon the nature of the covalent bond, the atoms involved, the type of the bonds and the strength of the intermolecular interactions. The IR spectrum of a sample is, therefore, a biochemical fingerprint. It has been found that the most significant changes occur in the mid – IR spectral range 3-25 µm.

The IR spectra are well known for their sensitivity to composition and three-dimensional structure of biomolecules, they allow measuring complex molecular vibrational modes. FTIR spectroscopy has become a widely used analytical tool in the biomedical sciences to characterize a variety of specimens, e.g., microorganisms, and isolated cells of the immune system, body fluids and tissues. The architectural
changes in the cellular and sub-cellular levels developing in abnormal tissue, including a majority of cancer forms, manifest themselves in different optical signatures, which can be detected in infrared spectroscopy. The molecular vibrational modes, which are responsible for IR absorption spectra, are characteristic of the biochemistry of the cells and their sub-cellular components.

The main objectives of this study are to evaluate the potential of the new technology of FTIR microspectroscopy (FTIR-MSP) in the field of medicine. Therefore we characterize different types of cells in cultures. Our main goal of using FTIR-microspectroscopy in human colonic tissues is to examine the potential of this technique to differentiate between normal, premalignant and malignant types of tissues. The systems we have measured using FTIR-MSP are:

1. Normal and H-ras transfected murine fibroblasts (Balb/3T3).
2. Normal and malignant mouse fibroblasts (NIH/3T3) transformed by retrovirus infection.
3. Normal primary rabbit bone marrow cells (BM) and bone marrow cells transformed (BMT) by murine sarcoma virus (MuSV).
4. Normal, premalignant (polyp) and malignant human colonic tissues from different patients, using microscopic infrared technique.

Conclusions of this work: The absorption of normal cells was found to be higher than the malignant and premalignant (in the case of colon) ones in the spectral range 600-3200 cm\(^{-1}\), when normalization to the amide I intensity was used. The differences in the primary cells were much higher than cell lines maybe because they replicate slowly (unlike the “normal” cell line.)

Phosphate contents were higher in normal cells relative to cancerous cells in all cell lines. An increase in the RNA/DNA ratio was observed for the cancerous cells, which correlates with the increased transcriptional activity expected for the cancerous cells.

We found significant differences between DNA absorption spectra of normal and cancer cells derived from NIH/3T3 cells (mouse fibroblast cells). Cluster analysis showed that it was possible to differentiate between normal and transformed cells with 100% accuracy in the rabbit bone marrow fibroblasts.
The results of Linear Discriminant Analysis (LDA) showed that the normal and malignant cells in colon tissues could be identified with 89% accuracy. The results show the influence of various independent factors such as age, sex and grade of malignancy. Our results suggest that among the above three factors, age and grade of malignancy have significant effect on the metabolites level, but sex has only minor effect on the measured spectra.

By employing a combination of wavelet features and Artificial Neural Network (ANN) based classifier, we were able to classify the different cell types such as normal, adenomatous polyp and cancerous in a given tissue sample. The percentage success of the classification was 89, 81 and 83 for normal, adenomatous polyp and malignant types respectively. Comparison of the proposed method with pathological method has also been discussed.

2) Experimental and Biological Background

2.1 Introduction

Air, water supplies foods, almost everything in use can be contaminated with chemicals having the potential of inducing cancer. The carcinogens can be organic or inorganic in nature. The polycyclic aromatic hydrocarbons such as Benz (a) anthracene and Benz (a) pyrene are classic examples of industrial carcinogens [1]. Various industrial processes using chromates, nickel and arsenic present a higher risk in carcinogenesis [2]. The molecular target of the chemical carcinogens is the nucleic acids, DNA and RNA in which their structural properties are changed drastically.

Cancer has been recently recognized as a genetic disease, in which a series of mutations, which can be inherited or acquired, accumulate with time and transform the cells into malignant phenotype. Causative agents include chemical and environmental carcinogens and tumor promoters, including radiation, transformation by DNA viruses or RNA retroviruses, and genetic inherited predisposition, and defective DNA repair mechanisms [3, 4, 5, 6]. Normal cells transform into malignant by activation of oncogenes, derived from mutated growth-controlling genes, encoding growth factors, receptors, intracellular signal transducers, nuclear transcription factors and cell cycle control proteins, and or inactivation of tumor-suppressor genes, (P5) in the case of colon cancer.
Early diagnosis appears to be an essential requirement for successful treatment of cancer, and tissue/cell analysis is the key to diagnosis in oncology. The analysis is traditionally performed in vitro: a biopsy is performed (or a sputum sample or swab is collected) and tissue sections are microscopically assessed by a pathologist after staining. The results are then related to an in vivo condition. In general, optical spectroscopy and spectroscopic micro-imaging offer a unique potential for the development of novel diagnostic tools in medicine, and one such important use is to distinguish between cancerous from non-cancerous states of the tissue and other pathologies. The underlying physical basis for the various optical approaches is that an optical spectroscopic signature of the tissue is influenced by the tissue biochemical composition and its cellular structure.

Infrared (IR) spectroscopy is well known for its uniqueness as a non-destructive method in identifying vibrational structure of various materials. The spectra allow measuring complex molecular vibrational modes. Various biomolecular components of the cell give a characteristic IR spectrum, which is rich in structural and functional aspects [7, 8]. One of the most promising applications of the IR-based techniques, which have become possible, now, is in biomedicine. IR spectroscopy can detect and monitor characteristic changes in molecular composition and structure that accompany transformation from normal to cancerous state [8, 9, 10, 11, 12, 13]. This could be done in the early stages of malignancy (e.g., polyp which is called pre-malignant), which are not yet evident in standard methods [14, 15, 16]. IR spectroscopy opens new and modern areas of medical research, as it causes no damage to the cells.

In the past few years, FTIR has been used in the diagnosis of cancer. Gao T., [17] has carried out the FTIR study of human breast, normal and carcinomal tissues. They report that their method of analysis results in nearly 100% diagnostic accuracy of carcinomal tissues from normal ones. Also the diagnosis of lung cancer was done using FTIR by measuring the ratio of the peak intensities of the 1030 cm\(^{-1}\) and 1080 cm\(^{-1}\) bands (originated mainly in glycogen and phosphodiester groups of nucleic acids respectively) which differs greatly between normal and lung cancer samples. The grading of lymphoid tumors could be achieved by FTIR microscopy. The examples mentioned above clearly suggest that FTIR can be a powerful tool in the
diagnosis of cancer. The main advantages of this technique are simplicity, quick results and economic viability.

2.2 Fourier Transform Infrared Spectroscopy (FTIR)

2.2.1 The electromagnetic spectrum

The spectrum of the electromagnetic waves is divided into different types as shown in Figure 2.2.1.

1. **Gamma radiation**: are generated by radioactive atoms and in nuclear explosions, and are used in many medical applications.

2. **X rays**: are high-energy waves, which have great penetrating power and are used extensively in medical applications and in inspecting welds.

3. **Ultra violet (UV)**: electromagnetic radiation at wavelengths shorter than the violet end of visible light, (400 nm to 10 nm), and are used in fluorescence.

4. **Visible light**: Electromagnetic radiation at wavelengths, which the human eye can see. We perceive this radiation as colors ranging from red (longer wavelengths; ~700 nanometers) to violet (shorter wavelengths; ~400 nanometers.)

5. **Infrared**: Infrared radiations are electromagnetic waves whose wavelengths are longer than the red end of visible light and shorter than microwaves (roughly between 1 and 100 microns). Infrared spectrum is divided into three regions: far infrared (in the wavenumber range 400-100 cm\(^{-1}\)), mid infrared region (in the wavenumber range 4000-400 cm\(^{-1}\)), and the near infrared (in the wavenumber range 14285-4000 cm\(^{-1}\)), where
The wavenumber is \(1/\lambda\) (cm). The majority of infrared applications in biological samples employ the mid infrared region [18].

6. **Microwave:** has a longer wavelength (between 1 mm and 30 cm) than visible light.

7. **Radio:** has the lowest frequency, the longest wavelength (less than a centimeter to tens and hundreds of meters), and is produced by charged particles moving back and forth.

Table 2.2.1 gives approximate wavelengths, frequencies, and energies for selected regions of the electromagnetic spectrum.

**Table 2.2.1:** summary of the electromagnetic radiation.

<table>
<thead>
<tr>
<th>Region</th>
<th>Wavelength (centimeters)</th>
<th>Wavenumber (cm(^{-1}))</th>
<th>Frequency (Hz)</th>
<th>Energy (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radio</td>
<td>&gt; 10</td>
<td>&lt; 0.1</td>
<td>&lt; 3 \times 10^9</td>
<td>&lt; 10^{-5}</td>
</tr>
<tr>
<td>Microwave</td>
<td>10 - 0.01</td>
<td>0.1 - 100</td>
<td>3 \times 10^9 - 3 \times 10^{12}</td>
<td>10^{-3} - 0.01</td>
</tr>
<tr>
<td>Infrared</td>
<td>0.01 - 7 \times 10^{-5}</td>
<td>100 - 14285.7</td>
<td>3 \times 10^{12} - 4.3 \times 10^{14}</td>
<td>0.01 - 2</td>
</tr>
<tr>
<td>Visible</td>
<td>7 \times 10^{-5} - 4 \times 10^{-5}</td>
<td>14285.7 - 25000</td>
<td>4.3 \times 10^{14} - 7.5 \times 10^{14}</td>
<td>2 - 3</td>
</tr>
<tr>
<td>Ultraviolet</td>
<td>4 \times 10^{-5} - 10^{-7}</td>
<td>25000 - 10^{9}</td>
<td>7.5 \times 10^{14} - 3 \times 10^{17}</td>
<td>3 - 10^{4}</td>
</tr>
<tr>
<td>X-Rays</td>
<td>10^{-7} - 10^{-9}</td>
<td>10^{-7} - 10^{-9}</td>
<td>3 \times 10^{17} - 3 \times 10^{19}</td>
<td>10^{3} - 10^{5}</td>
</tr>
<tr>
<td>Gamma Rays</td>
<td>&lt; 10^{-9}</td>
<td>&gt; 0^9</td>
<td>&gt; 3 \times 10^{19}</td>
<td>&gt; 10^{5}</td>
</tr>
</tbody>
</table>

### 2.2.2 Vibrational Spectroscopy

Light can interact with tissue in various manners, absorbing, scattering, reflecting etc...[19]. The chemical bonds vibrate when the matter absorbs IR radiation. Thus, the existence of chemical bonds is a necessary condition for infrared absorbance to take place. A molecule absorbs energy, when the intermolecular distance of two or more atoms changes. This is the vibrational energy. There are two oscillations of the atoms, which correspond to the normal modes of vibration: stretching and bending. Stretching is a symmetric or antisymmetric rhythmical
movement along the bond axis. The bending vibration occurs when the bond angle between two atoms or movement of a group of atoms might change, relative to the remainder of the molecule. These motions are known as scissoring, wagging, rocking, and twisting. Figure 2.2.2 shows the stretching and bending modes [18].

![Diagram of stretching and bending modes](image)

**Figure 2.2.2:** Typical stretching and bending vibrational modes [18].

The molecule’s absorbance of infrared radiation occurs under certain conditions: the radiation should have energy identical to one of the transitions between the discrete energy levels of the molecule. Moreover, there should be a change in dipole moment. For example, when two oxygen atoms in carbon dioxide symmetrically move in or out, no infrared radiation is absorbed because there is no change in the dipole moment. The symmetric stretch is therefore infrared inactive. However, when the oxygens both move left or right, the antisymmetric stretch, there is a change in the dipole moment, and infrared radiation is absorbed at 2350 cm\(^{-1}\). The vibration of diatomic molecule can be considered as two masses connected by a massless spring, which obeys Hooke’s law. The displacement of the two atoms from the center of mass is given by

\[
\mu \frac{d^2R}{dt^2} = -kR
\]

where \(R\) is the total distance between the two atoms, and \(\mu\) is the reduced mass:

\[
\mu = \frac{m_1m_2}{m_1 + m_2}
\]
A quantum mechanical treatment [20] shows that the energy levels are given by

\[ E_{\text{vib}} = (n + 1) h \nu \]  

2.2.2.3

where \( h \) is Plank’s constant, \( n \) is the quantum number, which has only positive integers. In order to absorb a photon and to be excited to higher vibrational state, the molecule energy difference should be equal to the photon energy. This condition could be achieved if the quantum number changes by \( \pm 1 \). This condition is known as the selection rule for molecule vibration [19, 21]. The selection rules for vibrational and rotational transitions are [20]:

\[ \Delta n = \pm 1, \Delta l = \pm 1 \text{ and } \Delta m = \pm 1 \text{ or } 0. \]  

2.2.2.4

For vibrational transitions, the matrix element is calculated as:

\[ \varepsilon_{n \rightarrow m} = \left| \psi_m^* \hat{\mu} \psi_n dr \right| \]  

2.2.2.5

where \( \mu \) represents the difference between the dipole moment of the ground and excited state. The dipole moment can be written as a Taylor series expansion:

\[ \mu(\vec{r}) = \mu_0 + \frac{d\mu}{dr} r + \frac{1}{2} \left[ \frac{d^2\mu}{dr^2} r^2 \right] + \ldots \]  

2.2.2.6

According to the characteristics of the wave function \( \psi \) [20], the contribution of the first component of the dipole moment in equation 2.2.2.6 to the matrix element is zero. In order to have non-zero matrix element there must be a change in the dipole moment during transition (\( d\mu/dr \neq 0 \)).

The absorbance of the functional groups, known as chemical structural fragments within molecules absorb infrared radiation in the same wavenumber range. This absorbance occurs regardless of the structure of the rest of the molecule that the functional group is in [18, 22]. For instance, the C=O stretch of carbonyl group occurs at \( \sim 1700 \text{ cm}^{-1} \) in ketones, aldehydes, and carboxylic acids.

The infrared spectroscopy is a useful chemical analytical tool due to the correlation between the wavenumbers at which a molecule absorbs infrared radiation and its structure. According to this correlation, the structure of unknown molecules could be identified from its infrared spectra.
The normal modes of vibrations for molecules with \( N \) atoms are given by \( 3N-5 \) for linear molecules and \( 3N-6 \) for nonlinear molecules. For example, a tri atomic, angular molecule like \( \text{H}_2\text{O} \) produces 3 normal vibrations: one symmetrical, one asymmetric stretching vibration and a bending vibration [23] while \( \text{CO}_2 \), which is a linear molecule, has 4 normal vibrations.

Infrared spectra can provide chemical structures as well as quantitative information, such as the concentration of a molecule in a sample. Table 2.2.2 shows some chemical structural fragments and their frequency range.

**Table 2.2.2:** characteristic infrared absorption frequencies.

<table>
<thead>
<tr>
<th>Bond</th>
<th>Compound Type</th>
<th>Frequency range, cm(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-H</td>
<td>Alkanes</td>
<td>2960-2850(s) stretch</td>
</tr>
<tr>
<td></td>
<td>CH(_3) Umbrella Deformation</td>
<td>1470-1350(v) scissoring and bending</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1380(m-w) - Doublet - isopropyl, ( t )-butyl</td>
</tr>
<tr>
<td>C-H</td>
<td>Alkenes</td>
<td>3080-3020(m) stretch</td>
</tr>
<tr>
<td></td>
<td>Aromatic Rings</td>
<td>1000-675(s) bend (many bands)</td>
</tr>
<tr>
<td></td>
<td>Phenyl Ring Substitution Bands</td>
<td>870-675(s) bend</td>
</tr>
<tr>
<td></td>
<td>Phenyl Ring Substitution Overtones</td>
<td>2000-1600(w) - fingerprint region</td>
</tr>
<tr>
<td>C-H</td>
<td>Alkynes</td>
<td>3333-3267(s) stretch</td>
</tr>
<tr>
<td></td>
<td></td>
<td>700-610(b) bend</td>
</tr>
<tr>
<td>C=( \text{C} )</td>
<td>Alkenes</td>
<td>1680-1640(m,( w )) stretch</td>
</tr>
<tr>
<td>C=( \text{C} )</td>
<td>Alkynes</td>
<td>2260-2100(w, sh) stretch</td>
</tr>
<tr>
<td>C=( \text{C} )</td>
<td>Aromatic Rings</td>
<td>1600, 1500(w) stretch</td>
</tr>
<tr>
<td>C-O</td>
<td>Alcohols, Ethers, Carboxylic acids, Esters</td>
<td>1260-1000(s) stretch</td>
</tr>
<tr>
<td>C=O</td>
<td>Aldehydes, Ketones, Carboxylic acids, Esters</td>
<td>1760-1670(s) stretch</td>
</tr>
<tr>
<td>O-H</td>
<td>Monomeric -- Alcohols, Phenols</td>
<td>3640-3160 (s,br) stretch</td>
</tr>
<tr>
<td></td>
<td>Hydrogen-bonded -- Alcohols, Phenols</td>
<td>3600-3200(b) stretch</td>
</tr>
<tr>
<td></td>
<td>Carboxylic acids</td>
<td>3000-2500(b) stretch</td>
</tr>
<tr>
<td>N-H</td>
<td>Amines</td>
<td>3500-3300(m) stretch</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1650-1580 (m) bend</td>
</tr>
<tr>
<td>C-N</td>
<td>Amines</td>
<td>1340-1020(m) stretch</td>
</tr>
<tr>
<td>C≡N</td>
<td>Nitriles</td>
<td>2260-2220(v) stretch</td>
</tr>
<tr>
<td>NO(_2)</td>
<td>Nitro Compounds</td>
<td>1660-1500(s) asymmetrical stretch</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1390-1260(s) symmetrical stretch</td>
</tr>
</tbody>
</table>

\( v \) - variable, \( m \) - medium, \( s \) - strong, \( br \) - broad, \( w \) - weak
2.2.3 Introduction to FTIR

The invention of Michelson interferometer in 1880 [24] made the development of FTIR possible. IR spectroscopy was impractical in the past because it took a long time to convert an interferogram into a spectrum. The invention of the “Fast Fourier Transform” FFT [25] and the revolution in computer power made the use of this method plausible. Using this method on biological samples is new and quickly expanding technique. Infrared spectral differences between healthy and cancerous human colon [26, 27], breast [17] and cervical [28] cells have been discovered, and discussed in the literature.

2.2.4 Advantages of FTIR

1) The signal to noise ratio (SNR) determines its ultimate performance. SNR is calculated as the ratio of the peak intensity of a feature in an infrared spectrum to the level of noise at some baseline point nearby as shown in Figure 2.2.3. For this peak the signal to noise ratio is 18.

![Figure 2.2.3: An isolated peak and associated baseline noise. The signal measured at 2228 cm\(^{-1}\) is 0.45 and the noise measured at 2916 cm\(^{-1}\) is 0.025. The SNR for this spectrum region is 18 [22].](image)
In our measurements, the spectra were very smooth (SNR was several hundred), and the signal to noise ratio was calculated using the SNR function in the OPUS software. This function calculates the SNR of a spectrum over a given spectral range. In the mid infrared, the SNR is calculated in the range 2100-1900 cm\(^{-1}\) according to BRUKER. Two S/N values can be calculated: RMS (Root Mean Square) and PP (Peak to Peak). In both cases, a quadratic parabola is fitted to the spectrum in the region of interest. This curve defines the (nominal) signal. RMS is calculated as the root of the mean square of deviations (i.e., the standard deviation). PP noise is the peak-to-peak excursions (maximum - minimum) in the y-direction. The (mean) nominal signal is divided by the newly calculated noise values and yields the SNR. A high SNR instrument is more sensitive, and applicable to more kinds of samples. Moreover, it allows to measure the absorbances more accurately than an instrument with low SNR does. A real spectral feature’s intensity must be at least 3 times than that of the noise. If it is less than that, it should be ignored [22].

There is no need to use a slit or another aperture device in FTIR spectrometry; thus, its throughput (all the radiation passes through the sample and strikes the detector at once in an FTIR spectrometer) is high, reducing the measurement time.

With the same spectral resolution and recording time [29]:

\[
\frac{(S/N)_{FT}}{(S/N)_{G}} \approx 200 \tag{2.2.4.1}
\]

where

(S/N)\(_{FT}\) is the signal to noise ratio in FTIR spectrometer.
(S/N)\(_{G}\) is the signal to noise ratio in grating spectrometer.

The FTIR can acquire a spectrum rapidly and simultaneously (BRUKER makes 128 scans in about 1 minutes, with 4 cm\(^{-1}\) resolution). Thus, multiple scans of the same sample can be added together. The multiple scans increase the SNR [30].

\[
\frac{(S/N)_{FT}}{(S/N)_{G}} = \sqrt{N} \tag{2.2.4.2}
\]

where \(N\) is the number of scans.

2) FTIR can analyze small samples of the order of nano grams.
3) It is easy to calibrate the spectrometer by using a reference laser (HeNe) to measure optical path difference (this will be discussed later in section).

4) FTIR is a noninvasive method, it is simple to use, quick and economic.

5) The spectral resolution is the same throughout the entire spectral range.

### 2.2.5 Limitations of FTIR

Limitation of infrared spectroscopy:

1. It cannot detect atoms or monoatomic ions because single atoms do not contain chemical bonds, and do not possess any vibrational motion. Consequently, they absorb no infrared radiation. Homonuclear diatomic molecules—molecules comprised of two identical atoms, such as N₂ and O₂—do not absorb infrared radiation.

2. The spectra obtained from samples are complex, and difficult to interpret because it is hard to know which bands are from which molecules of the sample.

3. Its usage in aqueous solutions is also difficult to analyze by means of infrared spectroscopy. Water is a strong infrared absorber in specific wavenumber ranges, thus it masks regions of the sample spectrum.

4. FTIR spectrometers are a single beam technique. The samples and the background are measured at different times. In order to eliminate the instrumental and environmental contributions to the spectrum, the sample spectrum is divided by the background spectrum. However, spectral artifacts can appear in the sample spectrum as a result of instrumental or the environmental changes of water vapor and carbon dioxide concentration during the time between the sample and background. The water vapor in the air (humidity) absorbs mainly in the regions 1270-2000 cm⁻¹ and 3200-4000 cm⁻¹)

### 2.2.6 Michelson Interferometer

The interferometer is the heart of our BRUKER Equinox 55 FTIR spectrometer. Basically, it works according to Michelson interferometer. Michelson interferometer takes a beam of light, splits it into two beams, and makes one of them travel a different distance than the other [31]. The optical path difference, δ, is the
difference in distance traveled by these two beams. It is the major component of most FTIR spectrometers. A diagram of a Michelson interferometer is shown in Figure 2.2.4.

![Michelson Interferometer Diagram](image)

**Figure 2.2.4:** An optical diagram of Michelson interferometer.

The beamsplitter is designed to reflect half the radiation that fall onto it, and transmit half of it, when an infrared light beam falls on it (this will be discussed later in section 2.2.9.3). The transmitted light strikes the fixed mirror, and the reflected light strikes the moving mirror. After reflecting off their respective mirrors, the two light beams interfere at the beam splitter, and then leave the interferometer to the sample compartment and interact with the sample and continue to the detector.

*Zero path difference* (ZPD) occurs when the reflected and the transmitted beam travel the same distance.

Light reflected by the moving mirror travels more than the light reflected by the fixed one. The *mirror displacement* is the distance that the mirror is moved relative to ZPD, and is denoted by $\Delta$. The relationship between mirror displacement and optical path difference is

$$\delta = 2\Delta$$  \hspace{1cm} 2.2.6.1

When using a monochromatic light source, such as laser, of wavelength $\lambda$, the recombined beams, reflected off the fixed and moving mirrors, on the beamsplitter, will be in phase. Since they move the same distance, they will arrive the sample also in phase. The two beams of light interfere. The result will be a light beam of double
amplitude (constructive interference) Figure 2.2.5, if the interferometer is in ZPD. This constructive interference also happens when:

\[ \delta = n^* \lambda \]  \hspace{1cm} 2.2.6.2

where \( n \) is any integer with the values \( n = 0, 1, 2, 3 \ldots \).

If the path difference

\[ \delta = (n +1/2)^* \lambda \]  \hspace{1cm} 2.2.6.3

where \( n \) is any integer with the values \( n = 0, 1, 2, 3 \ldots \) a destructive interference occurs, and the amplitude for each beam cancels that of the other as can be seen in Figure 2.2.5.

![Figure 2.2.5: Destructive and constructive interference.](image)

A combination of constructive and destructive interference occurs, making the light beam intensity somewhere very bright and very weak when the situation is between those given in equations 2.2.8 and 2.2.9.

The infrared radiation intensity increases and decreases smoothly if the velocity of the mirror is constant. When leaving the interferometer, the light goes through the sample compartment and focuses upon the detector, which measures the light intensity variation with optical path difference, as cosine wave. The fundamental measurement obtained by the FTIR spectrometer is the interferogram, which is a plot of detector response versus optical path difference. The interferogram is Fourier transformed to give a spectrum as described later.

A scan is generated when the mirror moves once to and from. Thus, a complete interferogram is generated.

The modulation frequency, denoted by the number of times per second the light switches between dark and light, is given by the following equation:
where $F_\nu$ is the modulation frequency, $V$ is the moving mirror velocity in cm/sec, and $W$ is the wavenumber of the light in the interferometer measured in cm$^{-1}$. A typical interferogram from our broadband infrared source is given in Figure 2.2.6. A Fourier transform is performed on the interferogram in order to get the spectrum.

![Interferogram of a broadband infrared source](image)

**Figure 2.2.6:** The interferogram of a broadband infrared source

Because all the wavelengths have constructive interference at ZPD, a sharp high intensity spike, known as *centerburst*, occurs there as shown in Figure 2.2.6. The interferogram’s intensity drops off rapidly as the mirror moves away from ZPD into the wings as a result of destructive interference, because the waves are out of phase.

In order to reduce the random noise and to increase the signal to noise ratio, interferograms are added together in an FTIR- a process known as coadding. The relationship between SNR and the number of scan $N$ is

$$\text{SNR} \propto (N)^{1/2} \quad 2.2.6.5$$

Increasing the number of scan is not illimitable. Beyond a certain number depending on the specific FTIR being used, it does not improve SNR. Drift in instrument response can contribute to noise in the spectrum in long measurements.
2.2.7 From Interferogram to Spectrum

A spectrum is calculated by Fourier transform of the interferogram. Ideally, the limits for Fourier transform integral should be plus and minus infinity.

The essential equations relate the light intensity falling on detector I (t), to the spectral intensity B (ν) are:

\[ I(t) = \int_{-\infty}^{+\infty} B(\nu)e^{-i2\pi\nu t} d\nu \]  \hspace{1cm} (2.2.7.1)

\[ B(\nu) = \int_{-\infty}^{+\infty} I(t)e^{-i2\pi\nu t} dt \]  \hspace{1cm} (2.2.7.2)

where \( \nu \) is the wavenumber. Equation 2.2.7.1 shows the variation in power density as a function path difference, which is an interference pattern. Equation 2.2.7.2 shows the variation in spectral intensity as a function of wavenumber.

Practically, the mirror is limited by the distance of its movement. Therefore, the signal is truncated at some finite time, resulting in affecting the spectrum of the absorbance bands of the infrared spectrum. Also, sidelobes- sinusoidal modulations in the baseline- surround the absorbance bands. In order to suppress these “feet” or lobes, we have to multiply the interferogram by an apodization function before the Fourier transformation of the interferogram. Reduction of spectral resolution is a side effect of using apodization functions. An example of this effect is shown in Figure 2.2.7.

![Figure 2.2.7: The spectrum of water vapor with boxcar apodization (bottom), and with a “medium Beer-Norton” apodization function (top).](image)
Although the peak is remarkably widened in comparison with the bottom spectrum, there is no sidelobes in the spectrum’s baseline. We should choose the apodization functions suitable to our goals; because they differ from each other in the way they suppress sidelobes, and their degrading of resolution. For quantitative analysis, the “medium Beer-Norton” function gives good results [32]. In our measurements we used the Blackman-Harris 4-term apodization function, which gave the best results.

The Fourier transform of an interferogram, of polystyrene measured at our spectrometer, produces a plot of infrared intensity versus wavenumber (cm\(^{-1}\)) as shown in Figure 2.2.8. A single beam spectrum obtained without a sample is called background spectrum. Figure 2.2.9 shows infrared spectrum of a background. A background spectrum must always be run before taking measurements in order to eliminate the instrumental and environmental contribution to the spectrum by ratioing the sample single beam spectrum against the background spectrum.

![Figure 2.2.8: A single beam infrared spectrum of polystyrene.](image)

The transmittance \( T \) can be calculated as:

\[
T = \frac{I}{I_0} \tag{2.2.7.3}
\]

where \( T \) is the transmittance, \( I_0 \) is the intensity entering the sample, and \( I \) the intensity passed through the sample at each wavenumber.
The absorbance spectrum can be calculated from the transmittance spectrum using the following equation.

\[ A = -\log_{10} T \]  

2.2.7.4

where, \( A \) is the absorbance.

**Figure 2.2.9:** A single beam background spectrum from air.

Figure 2.2.10 and Figure 2.2.11 show a transmittance and absorbance spectrum respectively of polystyrene. The chemical structure of polystyrene is shown below:

\[
\text{CH}_2\text{CH}_2\text{n}
\]

-CH

\[
\text{苯}
\]
Figure 2.2.10: Transmittance spectrum of polystyrene.

Figure 2.2.11: Absorbance spectrum of polystyrene.
2.2.8 Resolution and Signal to Noise Ratio

The spectral resolution determines the accuracy of separating different wavelength of radiation in the absorption spectrum. High resolution makes it possible to identify the exact location of narrow absorbance bands in addition to reducing spectral overlap between different bands. For example, a $4 \text{ cm}^{-1}$ resolution spectrum contains a data point every $4 \text{ cm}^{-1}$. Increasing the resolution causes other problems. For example, spectral noise increases as a result of intensifying resolution, making the analysis less precise.

Several factors influence the resolution in an FTIR spectrometer. The optical path difference increases the resolution of the spectrometer.

$$\text{Resolution} \propto \frac{1}{\delta}$$  \hspace{1cm} 2.2.8.1

where $\delta$ is the optical path difference.

The relationship between SNR and resolution is:

$$\text{SNR} \propto \text{Resolution}$$  \hspace{1cm} 2.2.8.2

A resolution of $4 – 8 \text{ cm}^{-1}$ will easily resolve most condensed phase sample bands [22].

According to Jaakkola [33] the signal to noise ratio of FTIR spectrum depends on the following factors:

1) Specific detectivity of the detector at a given wavenumber and modulation frequency.
2) Total spectrometer efficiency.
3) IR source temperature and emissivity.
4) Measurement time.
5) Spectral resolution
6) Optical throughput of the spectrometer.
7) Type of the IR detector (DTGS, MCT, etc.).

2.2.9 FTIR Hardware

2.2.9.1 Interferometer

The modulator system is the most important part of the FTIR spectrometer. Generally the name of spectrometer is determined according to the modulator [29].
Tilting the moving plane mirror during scan is the biggest disadvantage of Michelson interferometer. The tilting angle of the moving mirror should be less than about $\lambda/8D$, where $\lambda$ is the wavelength of the radiation under study and $D$ is the diameter of the moving mirror. This makes it very difficult to make the mirror move precisely [29].

Vibration and ambient temperature changes lead to modulation fluctuations in the Michelson interferometer, which, in their turn, cause errors and instability in the measured spectrum. This instability of the measured spectrum cuts down analytical sensitivity, accuracy and precision.

Different kinds of solutions have been developed with an attempt to solve these problems of the Michelson design. For example: cat’s eye reflectors, cube-corner mirrors and swinging interferometers [29].

2.2.9.2 IR Sources

IR-radiation broadband sources are the commonly used ones; they emit infrared radiation from far infrared to visible range. Their temperature is generally higher than 1200 °K. Broadband radiation sources for infrared spectrometers are simply blackbody radiators.

*Planck Radiation Law* describes the intensity of radiation emitted by unit surface area into a fixed direction (solid angle) from the blackbody as a function of wavelength for a fixed temperature. Figure 2.2.12 shows the intensity of Planck’s black body radiation at different temperature.

$$E(\lambda,T) = \frac{2hc^2}{\lambda^5} * \frac{1}{e^{\frac{hc}{\lambda kT}} - 1} \quad 2.2.9.1$$

where: $c \sim 3\times10^8$ m/s is the speed of light, $h \sim 6.626\times10^{-34}$ J*s is known as Plank's constant, $\lambda$ is the wavelength, $k$ is Boltzmann's Constant $k \sim 1.380\times10^{-23}$ J/°K and $T$ is the temperature.

The spectra of blackbody radiation as a function of wavelength are dominated by a peak at certain wavelength, which shifts to shorter wavelengths for higher temperatures. According to Wien’s Law,

$$\lambda_{\max} * T = 2900 \ (\mu\text{m} \cdot ^\circ\text{K}) \quad 2.2.9.2$$
Furthermore, the area under the curve increases quickly as the temperature increases. According to Stephan-Boltzmann’s Law,

$$F = \sigma T^4$$  \hspace{1cm}  \text{2.2.9.3}$$

where $\sigma = 5.7 \times 10^{-8} \text{ Wm}^{-2}\text{K}^{-4}$ and $F$ is energy flux (the power per unit area radiated from an object).

The only adjustable parameter of the source is temperature [29]. Globars (silicone carbide), Nernst glowers and nichrome coils are the most commonly used sources in the mid infrared region. Our spectrometer contains a Globar source, which is an air-cooled source that uses air currents to maintain its temperature. Globars do not require preheating. They consist of ceramic silicon carbide (SiC) and operate in the temperature range of 1200 – 1300 °C. They endure more mechanical exertion than nernst glowers. Globars’ intensity emission spectrum peaks at 1.92 μm, the emission intensity decreases remarkable at lower wavelengths, and the spectrum intensity is 75% of blackbody radiation. Globars’ are inexpensive, but they may not provide enough infrared intensity for some applications.

\textbf{2.2.9.3 Beamsplitter}

KBr itself does not split the infrared light because it is transparent to IR radiation. Consequently, beamsplitters consist of a thin coating of germanium

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2_12.png}
\caption{The emission intensity of blackbody radiation.}
\end{figure}
sandwiched between two pieces of Potassium bromide (KBr). The function of the Ge coating is to split the IR beam. This type of beamsplitter is almost universally used FTIR.

Ge on KBr, which is hard, and can be manufactured to the tolerances needed for a good beamsplitter, are usable in the range of 4000 to 400 cm\(^{-1}\), covering the mid-infrared very well. KBr is hygroscopic (it absorbs water from the atmosphere and fogs over)- which is a drawback.

Cesium iodide (CsI), transmitting from 4000- 200 cm\(^{-1}\), is another material used in the mid-infrared beamsplitters. The disadvantage of CsI is that it is soft and very hygroscopic, which means that it should be replaced more frequently.

The beamsplitter splits half of the radiation that impinges it at 45\(^0\) and transmits the other half [21].

2.2.9.4 Detectors

The infrared detector is a device measuring the infrared energy of the source, which has gone through the spectrometer. Radiation energy is transformed into electrical energy by these devices. The electrical energy can be processed to generate a spectrum [21]. Using a high sensitivity detector in the IR spectrometer is very important, because it determines the maximum possible SNR and the minimum recording time of the spectrum. Thermal detectors (thermocouples, bolometer and pyroelectric (DTGS)) and quantum detectors (MCT) are the two basic types used in the IR spectroscopy. An advanced spectrometer such as (BRUKER EQUINOX model 55) used in our study is equipped with both DTGS and MCT detectors for chamber measurements, and MCT detector for Microscopy. The wavenumber range and the sensitivity depend on the choice of the detector. In this work we used the sensitive MCT detector to obtain a good quality of spectra.

2.2.9.4.1 DTGS

The most commonly used material for the thermal detectors is DTGS (deuterated triglycine sulfate). Temperature changes due to IR radiation influence the dielectric constant of the DTGS material, and affect the detector capacitance. These changes in the capacitance as a function of temperature are measured as a voltage across the detector element [22, 34].
Thermal conductors work over a wide range of wavelengths including mid-infrared-region. They are generally slow with a time constant typically from 0.001 to 0.1 second. The voltage responsivity of a pyroelectric detector at frequency $f$ is roughly proportional to $1/f$. The operation range of the DTGS detectors equipped with KBr windows is from 4000 to 400 cm$^{-1}$ [22, 29]. These detectors are simple, inexpensive, and robust. They cannot be used in infrared Microscopy and gas chromatography (GC-FTIR) because they are not as sensitive as quantum detectors.

### 2.2.9.4.2 MCT

MCT (mercury cadmium tellurium (HgCdTe)) detector is a quantum detector. IR radiation excites the electrons to higher energy levels, and changes the electrical conductivity of the detectors. For example- in the n-type semiconductor, the electrons are excited from their valence band to the conduction bands. Electrons in the conduction are current carriers and they respond to applied voltage, increasing the electrical current.

The measured electric current is directly proportional to the number of infrared photons hitting the detector. Generally, liquid nitrogen or peltier element cools the MCT detector element for good sensitivity. Peltier cooled MCT detectors operate in the wavenumber range from 4000-900 cm$^{-1}$ [29]. Liquid nitrogen cooled MCT detector may operate from 6000 to 600 cm$^{-1}$. In our spectrometer the MCT detector was nitrogen cooled, and our measurement range was 4000 to 600 cm$^{-1}$.

The MCT detectors cost 5 to 10 times higher than DTGS detectors. Strong IR signals saturate the MCT detectors, that is, beyond certain intensity, the measured electrical current does not change. This can be a problem when doing quantitative analyses. Figure 2.2.13 shows the MCT detector in our FTIR spectrometer [BRUKER, Equinox 55].
Figure 2.2.13: MCT detector and Mounting plate (A) in our spectrometer.

2.2.9.5 Electronics

Two purposes make the He-Ne laser whose light is red and its wavelength is known precisely ($\lambda = 632.8$ nm) a very important component in every FTIR spectrometer. First, all infrared wavenumbers are measured relative to the He-Ne laser light line, which is determined precisely at 15798.637 cm$^{-1}$. Consequently, the wavenumber accuracy of most FTIRs is $\pm 0.01$ cm$^{-1}$ or better. One way to check laser misalignment is to take a spectrum of polystyrene film and measure the position of the peak at 1601 cm$^{-1}$. Its position should be constant.

The second purpose is to use the He-Ne to measure the optical path difference properly, by tracking the position of the moving mirror [22].

A microprocessor, which controls all operations of the spectrometer, is the main component of the electronics in the computer controlled FTIR spectrometers. The computer in FTIR spectroscopy does all the data manipulations. Detector preamplifier, main signal amplifier, an analog to digital converter (AD converter), a set of band-pass electronics filters which are used in order to determine the desired wavelength range and the power supply are other components of FTIR spectrometers.

One of the major sources of noise in FTIR spectrometers is clipping, which occurs when the centerburst of the interferogram overfills the ADC. When this happens, the ADC will truncate the interferogram, and thus part of it will not be
measured. To prevent clipping, we amplify the interferogram using a gain amplifier in order to adjust the interferogram to its optimum value. In order to measure the low intensity in the wings precisely, the wings are amplified.

2.2.10 Sampling Techniques

Transmission, reflectance, and photoacoustic are sampling techniques. We used the transmission technique for solid samples [18, 21, 22]. The transmission technique is always preferable over other techniques since it gives the best signal with high SNR.

2.2.10.1 Transmission technique

Transmission technique is the most popular technique in which the radiation passes directly through the sample. There are many advantages of this technique. First, it has a high SNR. Second, it could be used for solids, liquids, gases and polymers. Third, the tools that are used to prepare the samples are not expensive.

Thickness is a major disadvantage of this technique. It is impossible to measure samples that are thicker than 20 microns, because they have a large signal. Moreover, samples that are thinner than 1 micron cannot be measured by this technique, because they have weak absorbance that cannot be detected by this sampling technique.

2.2.10.2 Transmission Spectra of Solids

KBr pellets and mulls are the first major type of sampling in transmitting mode in solids. The first type is suited for powders, or samples that can be ground into a powder. The second type involves two ways of preparing a sample, cast films, and heat and pressure films.

2.2.10.3 Reflectance Techniques

In this technique, we measured the reflected IR radiation from the samples. There are several disadvantages. It requires very expensive special accessories, and the penetration depth of the radiation is not known accurately. Consequently, quantitative measurement cannot be performed by this technique. It has SNR lower than the transmission technique if they are used under the same conditions. Also, it cannot be used for gases.
There are some advantages of this technique. For example, there is no thickness problem, so we can save time in preparing the sample, and these techniques are also nondestructive.

There are many types of reflectance techniques, Diffuse Reflectance (DRIFTS), Quantitative DRIFTS, Attenuated Total Reflectance (ATR), and Specular Reflectance.

2.2.10.4 Infrared Microscopy (Microspectroscopy)

Coupling of the microscope to an infrared spectrometer is defined as microspectroscopy. In this technique, we use a special type of microscope known as (IR microscope) [35].

The microscope works in two modes: visible and infrared. We examine the seeded sample on the transmitting window (ZnSe in our case) in the visible mode. The selected microsample area can be varied as desired. After visual examination in visible light, we change the IR-microscope mode to IR mode. Then, we record the infrared spectrum of the selected microsamples (in transmission mode). This method is ideal for biological samples, because they are heterogeneous [36].

IR microspectroscopy provides the capability to obtain spectra on extremely small samples, as small as about 10 µm in diameter.

We performed our measurements in the transmission mode using IR microscope. The detector was MCT.

The sample was brought into focus by means of the stage adjustment after putting it at the center of the microscope stage. Then, using the substage condenser, we brought the circle adjustable aperture between the detector and the sample into focus. After that, we determine the sample size about 100 µm in diameter, and we took a single beam spectrum.

2.2.11 Spectral Initial Analysis

OPUS software, allows to do various spectral analysis steps as outlined in the following sections:
2.2.11.1 Post-Zerofilling

During the Fourier transformation a zerofilling is automatically carried out over the whole spectral range. However, this increases the total size of the data file. Using post zerofilling allows you to apply the interpolation once again for only the frequency range of interest, which keeps the size of your files small. Using zerofilling factor 2 doubles the number of spectrum points by interpolation.

We used the post-zerofilling function with factor of 2. Choosing factor 2 increases the number of points in the spectra and makes them smoother with better shapes. However, zerofilling should not be confused with an increase in spectral resolution.

2.2.11.2 Baseline Correction

There are many reasons that make the spectral baseline not flat like sample scattering, inappropriate choice of background, and instrument drift. We can correct sloping and curved baselines by using the baseline correction function to make them flat. Figure 2.2.14 shows an example of an absorption spectrum before and after baseline correction using “Rubberband” correction. The baseline correction is generated as follow: First, we generate a function (polynomial, or any curve) that parallels the shape of the baseline in the sample spectrum. Secondly, we subtract this function from the spectrum, yielding a spectrum without a baseline.

There are two ways to generate this function in the opus software, rubberband correction and scattering correction.

In the rubberband correction, we divided the spectrum into 64 equal size ranges to construct the baseline. For absorbance spectra, the minimum y-value of the ranges is determined. A polynomial function was fitted according to these minimum points. Then we subtract this function from the spectrum to extract the baseline corrected spectrum.

In the scattering correction, the baseline function is constructed in such a way that at each point the slope of the baseline must be negative for an absorbance-like spectrum. Figure 2.2.15 shows an example of baseline of an absorption spectrum.

We used the rubberband correction with 64 ranges -(we realized that increasing the ranges do not improve the baseline correction)- using the scattering
Figure 2.2.14: A typical baseline shape of one of our normal colonic absorption spectrum.

Figure 2.2.15: An absorption spectrum before and after base line correction using Rubberband correction of one of our normal colonic sample.
method, the baseline correction was bad in the low wavelength region in some of our spectra.

### 2.2.11.3 Smoothing

Smoothing is used on noisy spectra to reduce the noise level, so features that may have been hidden under the noise can be seen well. Our spectra are very smooth, so we did not use this option in the analysis.

### 2.2.11.4 Normalization

In order to compare spectra from different samples, we need to normalize them. There are three different spectral normalization methods in the OPUS software:

- **Min/Max normalization** - The spectrum is shifted so that the minimum occurring y-value is set to zero. The spectrum is changed in such a way that the minimum y-value becomes zero and the maximum y-value in the specific range is expanded in the y-direction to 2 absorbance units.

- **Vector normalization** –
  
The vector normalization is achieved using the following steps:

  1) Calculate the average y-value of the spectrum \( \overline{y} = \frac{\sum_{i=1}^{N} y_i}{N} \).

  2) Subtract the average \( \overline{y} \) from the spectrum to pull the middle of the spectrum to \( y = 0 \) \( (\tilde{y}_i = y_i - \overline{y}) \).

  3) Calculate the sum of the squares of all \( \tilde{y} \)-values \( (S = \sum_{i=1}^{N} \tilde{y}_i^2) \).

  4) Dividing the spectrum by the square root of this sum \( (x_i = \frac{\tilde{y}_i}{\sqrt{S}}) \). The vector norm of the resulting spectrum therefore equals 1 \( (\sum_{i=1}^{N} x_i^2 = 1) \).

- **Offset correction** - The spectrum is shifted so that the minimum occurring y-value is set to an extinction of zero.
We used the first two methods to normalize our spectra. It is difficult to say which method min-max or vector normalization is better. The normalization method should be selected according to the type of spectra and goals of the research.

In the min-max (Amide I) normalization, the intensity is positive in the whole range, while in the vector normalization, the intensity is “negative”. If we want to calculate metabolites such as integration, and ratios of intensity, then we cannot use vector normalization. Disadvantages of min-max normalizations are: the amide I contains also contributions from water, so it is sensitive to humidity, the normalization depends on a small range of the spectrum, and the whole spectrum is normalized according to it, while the vector normalization relies on the whole spectrum. Another disadvantage is that we cannot detect changes in amide I intensity due to differences in the samples.

2.3 Cancer

2.3.1 Introduction

Cancer is a title of more than 100 diseases. Most of the body cells repair and reproduce themselves similarly in spite of their different functioning and appearance. As long as this process takes place in an orderly manner, everything is under control. However, when it gets out of control, a lump, called a tumor, develops. There can be benign tumors, In-situ tumors or malignant tumors [37]. The rectum and colon, just like other organs of the body, consist of different kinds of cells. In the normal situation, cells divide to produce more cells when the body is in need for them. Benign tumors are not considered cancer. They do not threaten life, because they do not spread to other organs, and once they are removed, they rarely reappear. In-situ tumors look like cancer in their morphology. In general, they develop in epithelium. These small tumors remain in the epithelial layer. A malignant tumor consists of cancer cells that sometimes spread away from the original (primary) cancer to other organs through the lymphatic system and bloodstream causing damage to organs and tissues near the tumor. Worse is that when they get to another part of the body, they may divide forming a new tumor, called a secondary or a metastasis tumor [38].
Cancers are classified according to the tissue and cell type from which they arise. Carcinomas cancers are those developing in epithelial cells, while Sarcomas are cancers that develop in connective tissue or muscle cells. Benign tumors have also a set of names: an adenoma, for example, is benign epithelial tumor with a glandular organization. The different kinds of cancers have different causes and, consequently, different treatments.

Most of human cancers (approximately 90%) are carcinomas. This might be explained by two factors: cell proliferation mainly occurs in epithelia, and they are more frequently exposed to various forms of physical and chemical damage that favor the development of cancer [39]. Dedifferentiation or anaplasia denotes the loss of normal characteristics.

Biopsy is used by doctors to decide whether tumor is benign or cancerous by examining a small sample of cells under a microscope.

2.3.2 Oncogenes

There is a genetic damage, responsible for tumorigenesis, in most of the cancer cells. Cells are activated by an extracellular growth factor, which binds to its membrane. Consequently, certain reactions are triggered resulting in altered gene expression [40]. Some proteins, as well as small regularity molecules, are activated by the receptor. The activated proteins and regularity molecules transduce signals to the nucleus, and the cells start dividing. This consequence of successful communications dominates the growth of the cells. Growth inhibitory signals are generated to stop cellular proliferation from indefinite proceeding [41]. Many oncogenes are altered versions of the normal genes that control cell growth and function at every level of the growth regulation [40, 42, 43].

Proto-oncogenes code for cellular proteins that relay signals to a cell's nucleus, stimulating growth. These cellular proteins are responding to signals from other cells. Oncogenes are mutated forms of proto-oncogenes, whose functions are to encourage and promote the normal growth and division of cells. When proto-oncogenes mutate to become carcinogenic oncogenes, the result is excessive cell multiplication. The term oncogene itself is derived from the Greek word "oncos," meaning tumor. There are approximately 100 known oncogenes. There are different
groups of proto-oncogenes classified according to their normal functioning and homology sequence to other known proteins.

Proto-oncogenes are found at all levels of the different signal transduction cascades that control cell growth, proliferation and differentiation. Oncogenes encode proteins called oncoproteins, which lost important regulatory constraints on their activity, and they do not need external activation signals [41]. Oncoproteins associated with the inner surface of the cellular membrane are encoded members of the largest group of oncogenes that includes the src and ras families of gene types [41].

The process of activation of proto-oncogenes to oncogenes can include retroviral transduction or retroviral integration, point mutations, insertion mutations, gene amplification, chromosomal translocation and/or protein-protein interactions.

In our work the mouse fibroblast cells were transfected by H-ras oncogene from the human T24 bladder carcinoma [44]. Murine fibroblast cells were transformed into cancerous by the retrovirus MuSV, which was isolated from Clone 124 of TB (which are mouse fibroblast cells) [45]. In human colonic tissues, the cancerous tissues were obtained from different patients and the source of these tumors could be genetic, viral, chemicals, etc…

2.3.3 H- ras

In 30% of the tested spontaneous human tumors the activated oncogene ras is pronounced [46], and it is pronounced in more percentage (50%-70%) in specific tumors such as colon, pancreas and lungs cancers. The ras consists of a large super family of proteins (including Ha-ras, K-ras, N-ras) that affect many cellular activities such as cell growth, differentiation, secretion, and protein trafficking [47].

Ras oncogenes were the first non-viral oncogenes discovered. They were also found in sarcoma viruses before. These oncogenes encode the GTP binding proteins with GTPase activity.

Over-expression of ras and ras mutations was identified in various human tumors, like oral or head and neck squamous cell carcinoma, lung cancer and in pancreatic carcinoma [46, 48, 49, 50].
Ras oncogenes may play an early role in neoplasia, thus, there is a need for early detection of ras oncogenes in harboring cells. This was suggested by several reports of ras activation and mutations in benign pancreatitis in humans [50] and proceeding the onset of neoplasia in experimental models of carcinogenesis [51].

The p21 proteins, are 21 kDa (1000 mass unit) proteins, that are associated with the inner surface of the plasma membrane [52], encoded by ras appears to be involved in the regulation of cell proliferation and differentiation.

At the first stage of the cancer, the guanosine triphosphate (GTP)-bound form, which presents the activation conformation, is activated by these proteins [51]. Deactivation of GTP is performed by hydrolysis of GTP [53] and it is transformed into inactive guanosine diphosphate (GDP)-bound form [53]. The activation of this oncogene could be influenced by environmental factors such as carcinogenises [46] and U.V. radiation [54].

Mainly depending on common structure features, this super family of proteins is divided into six subfamilies [55].

Figure 2.3.1 shows a schematic diagram of ras GDP/GTP cycle. Normally, Ras p21 proteins are found in the GDP bound conformation (inactive). Activation of p21 is thought to be achieved by binding to an exchange protein GNRp (Guanine Nucleotide Releasing Proteins) and subsequent release of GDP. After dissociation of GDP, the nucleotide free ras protein binds GTP. This GTP-bound form then undergoes a conformational change into the active form, perhaps through interaction with GTPase-activating protein (GAP) [53].

Figure 2.3.1: Schematic diagram of ras GDP/GTP cycle [53].
After hydrolysis and subsequent conformational change into the inactive form, the cycle can be used again. Figure 2.3.1 [53]. GAPs are responsible for the stimulation of GTPase activity of ras [47]. The proteins GAP act on ras-GTP proteins and transform them into inactive form. The ras mutation causes the accumulation of the active form of ras-GTP, and prevents the ability of the GAP to transform the ras to its inactive form ras-GDP [47, 56]. As a result the mutant ras stays in its active form and thus enables permanent stimulation of signals. Because of that, these mutations cause cancerous tumors, and act through permanent activation of mutagenes signals, which are not dependent in the outside factor growth [46, 57, 58].

2.3.4 Retroviruses

2.3.4.1 Introduction

Viruses can induce malignant tumors. There are two different kinds of viruses: viruses with DNA genomes (e.g. papilloma and adenoviruses) and those with RNA genomes. Retroviruses are RNA viruses involved in different cancers such as leukemia and other malignant tumors in humans and animals [59, 60, 61, 62, 63, 64, 65, 66, 67, 68]. RNA tumor viruses are common but rare in humans. Human T-cell leukemia viruses (HTLVs), and human immunodeficiency virus (HIV) are the only currently known human retroviruses. Retroviral tumors are common in chickens, mice and cats.

2.3.4.2 Genome Structure

Retroviruses are divided into two major parts according to their genome structure: Leukemia viruses and Sarcoma viruses [69]. The leukemia viruses can replicate without transforming the cell, and they contain three genes env, pol, and gag. Sarcoma viruses transform normal cells into cancerous, but they cannot replicate, and they contain also onc oncogene that enables them to transform the cells. In order to replicate, they need the help of the leukemia viruses [70]. These genes that are parts of the Sarcoma viruses, are called V-oncs, and they include the genes, src, myc, ras, mos, sis. In our case the MuSV contains the mos gene [71].
2.3.4.3 Morphology

Retroviruses’ structure is illustrated in Figure 2.3.2. Retroviruses are envelope viruses, and consist of an inner core, the capsid, comprising nucleocapsid (NC) and capsid (CA) proteins, a membrane-associated protein shell composed of matrix protein (MA), and an outer lipid bilayer derived from cellular membranes with embedded viral transmembrane (TM) and surface (SU) glycoproteins.

![Diagram of Retrovirus Structure](image)

**Figure 2.3.2:** Schematic diagram showing the structure of a retrovirus. It includes host cell-derived lipid membrane, outer envelope, and transmembrane protein products of the env gene, and RNA genome [This figure is taken from ref. [72]].

The virus’ lipid bilayer envelope stems from host cell. Proteins of host cell origin, as well as those encoded by the env gene of the virus, are surrounded within this lipid bilayer envelope. Proteins encoded by viruses determine the host cell tropism of retrovirus. The outer envelope surrounds the envelope of the core. Molecules of protein P30 are in the inner side of the inner envelope. Inside the core exists a nucleo-protein complex, which is built from two molecules of the genome RNA that are bound to various type of tRNA from the host cell [101]. The viral genome is like mRNA so it can be used to produce viral proteins [104, 105].
2.3.4.4 Mode of transmission

Retroviruses can be transmitted in various ways as infections particles into extracellular space. It may enter the cell by direct fusion of cell and virus membrane [73] or by receptor mediated endocytosis [74], or perhaps both. After penetrating, the virus presumably undergoes uncoating process that releases capsid contents into the cytoplasm of the cell.

The cells are retrovirally transformed by two major mechanisms related to the life cycle of these viruses. First, a synthesis process of a proviral DNA copy of the viral RNA starts after a retroviral infection of a cell [75] (reverse transcriptase). After that, this DNA gets integrated into the genome of the host cell, which means that it remains being copied as the host genome is duplicated during the process of cellular division.

While replicating, retroviruses may get cellular sequences by means of erroneous recombination events. If the acquired cellular gene is a proto-oncogene, a gene involved in such functions as cellular homeostasis, growth control, etc., it can lead to malignancy by over expression under the control of strong viral promoters. It was found that this mechanism of provirus integration affects the c-myc proto-oncogene.

Second, complex retroviruses may encode genuine viral proteins (not acquired through recombination with cellular sequence) that can act oncogenically.

Other mechanisms that may contribute to oncogenesis include the direct stimulation of cell growth by viral env proteins or retrovirus-mediate immunosuppression by various ways [5, 76].

2.3.5 Tissues

Tissues are defined as cells with their ground substance acting together in the performance of a particular function. There are four primary tissue categories in human histology. These are epithelial, connective, muscle, and nervous [37]. Herein we discussed briefly each category:
**Epithelial**

Epithelial tissues form sheets that cover the surface of the body and line cavities, tubular organs and blood vessels. They have numerous cellular junctions, and tight adhesion, which are suitable for the epithelial tissue’s functions as a protective covering, absorption, secretion, sensation and contractility. They consist of closely aggregated cells with very little extracellular matrix. Whatever gets into or out of the human body goes through an epithelial layer [37].

There are different shapes of epithelial cells: columnar, cuboidal, and flattened squamous. They could be in the shape of single layer (simple), as in the lining of the alveoli of the lungs, or multiple-layer (stratified epithelia) as in the skin or lining of the mouth.

Adaptations at the luminal surface of the epithelial cells- like in the lining of the small intestine, which consists of hundreds of microvilli surrounded by glycoprotein coating- have two functions: they increase absorption by increasing the surface area, and they help in moving substances over the epithelial surface. The microvilli are covered by the plasma membrane and are approximately 1 micron high and 0.08 micron wide.

**Connective**

Connective tissues include cartilage, adipose, tendons, bone and ligaments. Their functions are cushioning, supporting and maintaining forms within the body. Protein fibers, ground substance and tissue fluid compose the extracellular matrix, which is the major element of connective tissue.

Fibers are made of collagen or elastin and are classified as collagen, reticular or elastic fibers. The different types are present in varying proportions dependent on the tissue type. Collagen consists of several types and is found in the human body. Fibroblasts make the fibers and ground substance and they constitute the major part of the connective tissue cells. Their cytoplasm is irregularly branched, and they have well developed endoplasmic reticulum, Golgi apparatus large and ovoid nucleus. They grow well in cell culture and they can divide many times in the growing plates. They rarely divide in adults’ connective tissues [37].
Muscle

Muscle is an excitable tissue, it can be stimulated mechanically, chemically or electrically to produce an action potential. Muscle cells contain a contractile mechanism that is activated by the action potential. There are several types of muscles: **Skeletal Muscle** is composed of cells, called fibers, which are specialized to contract or shorten in length. Skeletal muscle makes up most of the body's muscle and does not contract without nervous stimulation. **Smooth Muscle** is found in the walls of blood vessels, tubular organs such as the stomach and uterus, the iris, or associated with the hair follicles. It exists in the body as multiunit or visceral smooth muscle. It is not under voluntary control, each cell has one nucleus and it is displays automaticity in the visceral form. **Cardiac Muscle**, the heart is made of specialized muscle tissue with some similarities to both smooth and skeletal muscle [37].

Nerve

Nerve tissue is distributed throughout the body in a complex integrated communications network. In general, neurons consist of three parts; the cell body, dendrites and the axon. Neurons may be classified according to function. Sensory neurons are involved in the reception and transmission of sensory stimuli. Motor neurons send impulses to control muscles and glands. Other neurons, interneurons, act as go-betweens neurons as part of functional networks [37].

Stem Cells: A Primer

These are unique cells that can divide for indefinite periods in culture and give rise to specialized cells.

2.3.6 Colon Cancer

The large intestine, which is also called the large bowel (Figure 2.3.3) [77], consists of the rectum and the colon. Colon is the place where the waste materials of the digestive system gather. The adult’s large intestine is about 1.5 meters, while the small intestine is about 6 meters long. The end of the colon is called the rectum and it is close to the anus. Due to their strong relation, the cancer of these two parts are discussed under the same title, the colorectal cancer.

The inside layer of the small intestine consists of a large number of villi, which look as fingers covered with epithelial cells directed towards the lumen.
Crypts go down to the muscularis mucosa and they lie between the bases of the villi. Figure 2.3.4. [78].

Growths on the internal wall of the large intestine are the source of the colon and rectum tumors. They can be benign, known as polyps, or malignant, known as cancers. Unlike the benign polyps, the cancers invade the nearby tissues and spread to other parts of the body.

Figure 2.3.3: Histology of the large intestine (This figure is taken from ref. [78]).

Polyps are a tumor mass that stands out into the lumen of the gut Figure 2.3.5. [39], mainly, this term refers to lesions arising within the mucosa. Benign polyps do not threaten life, if they are removed early; otherwise they can develop into malignant tumors, which happens in most cases of large intestine cancers, as doctors believe [79]. A full recovery of cancer is almost impossible in case of metastasis in colorectal cancer.
Adenomatous polyp, or adenomas are polyps arising of proliferative dysplasia. Non-neoplastic polyps are generated by abnormal mucosal maturation, inflammation, or architecture. These polyps, like the hyperplastic polyp, are not potentially malignant.

According to epithelial architecture, adenomatous polyps are divided into three subtypes:

- **Tubular adenomas**: tubular glands
- **Villous adenomas**: villous projections.
- **Tubulo villous adenoma**: a mixture of tubular glands and villous projections.

From ref. [80] we know that the colon cancer is common in Western countries as well as in countries where the people have western diets. On the other hand, it is rare in Asian and African countries. It is the fourth cause of cancers among women and the third among men.

**2.3.6.1 Incidence**

Approximately 5% of the people suffer from colorectal cancer in their life. It is estimated that about 56,000 USA citizens die of it annually [79]. Recovery of colon cancer depends on the stage at which it is discovered [79]. It is believed that polyps become malignant in years. Thus, check-ups and early diagnoses are of great importance [79].

The cause of the large bowel cancer is unknown in most cases. There are several risk factors for large bowel cancer.

**2.3.6.2 Risk factors?**

Colorectal cancer is frequent among people over 50 [79]. There are several risk factors [80, 81, 82] like

- Taking much fat
- Family history of colorectal cancer and polyps
- Chronic ulcerative colitis
- Women who have had cancers of the breast, uterus or ovary may also have a higher risk of developing colorectal cancer.
2.3.6.3 How is it diagnosed?

The large bowel cancer is diagnosed [83] in several ways like:

**Sigmoidoscopy:** the doctor puts a thin tube containing a light and a lens into the rectum and sigmoid colon and examines whether there are tumors or polyps.

**Colonoscopy:** the doctor examines the lining of the whole colon by means of a camera attached to a long tube. He can also take a sample of the tissue for testing by means of a tiny instrument.

**Barium Enema:** the technicians take an X-ray of the patient after giving him/her a solution containing mineral barium. Usually, the doctors or technicians pump air in the tract to enlarge the colon and the rectum so that they can examine with better details.

**CT (Computerized Axial Tomography) Scan:** CT is a complicated X-ray that gives a more detailed picture of many layers and lasts about 10-30 minutes.

**MRI (Magnetic Resonance Imaging):** the patient lies still on a couch inside a long chamber for about half an hour and goes through a tunnel surrounded by a powerful magnet. A computer follows up magnetism and takes pictures of the tissues.

**Ultrasound:** a computer transforms the echoes that return from the patient’s into a sonogram image when very high frequency sound waves fall onto it.

**Further tests**

For further investigation, a biopsy is used in order to get an accurate diagnosis. This biopsy is sent to a pathologist who can evaluate the specimen for abnormal growth. Stagings of cancers are classified according to cell morphology and to where the cancer exists in the colon layers [84,85]. This issue is discussed next

2.3.6.4 Grading and staging of bowel cancer

Tumor grading is based on histological and cytological criteria, and it examines the differentiation level in tumors. Staging is examining the spread of cancer to other parts of the body in order to assess its progress in the patient [84].
Figure 2.3.4: (a) Diagram of the small intestine and enlarged villous. Identified in cross section are the layers of tissue: serosa, muscularis (note the absence of oblique muscles fibers), submucosa, and mucosa. The mucosa has modified to form villi, the finger-like projections [78]. (b) Diagram of the mucosal villous, submucosa, muscularis, and serosa [78].
Duke's classification system categorized patients into three stages: A, B, and C. Astler-Coller added a fourth stage (Stage D) [85]. Listed below is the modified Duke staging system.

Figure 2.3.5: Different kinds of polyps in the human intestine [39].
Table 2.3.3: Modified Duke Staging System [85]

<table>
<thead>
<tr>
<th>Modified Duke A</th>
<th>The tumor penetrates into the mucosa of the bowel wall but no further.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Modified Duke B</td>
<td><strong>B1</strong>: tumor penetrates into, but not through the muscularis propria (the muscular layer) of the bowel wall.</td>
</tr>
<tr>
<td></td>
<td><strong>B2</strong>: tumor penetrates into and through the muscularis propria of the bowel wall.</td>
</tr>
<tr>
<td>Modified Duke C</td>
<td><strong>C1</strong>: tumor penetrates into, but not through the muscularis propria of the bowel wall; there is pathologic evidence of colon cancer in the lymph nodes.</td>
</tr>
<tr>
<td></td>
<td><strong>C2</strong>: tumor penetrates into and through the muscularis propria of the bowel wall; there is pathologic evidence of colon cancer in the lymph nodes.</td>
</tr>
<tr>
<td>Modified Duke D</td>
<td>The tumor, which has spread beyond the confines of the lymph nodes (to organs such as the liver, lung or bone).</td>
</tr>
</tbody>
</table>

2.3.6.5 Treatment [83]

Surgery is the main treatment for cancer of the large bowel. It may be used either alone or in combination with radiotherapy and chemotherapy.

**Surgery**: it is the most commonly used treatment for colon cancer at early stage, and it is very effective in this case. However, removing colon cancer by surgery in an advanced stage will not cure it but can relieve symptoms only.

**Chemotherapy**: using chemotherapy reduces the possibility of the cancer to come back, and helps to prevent the spread of advanced cancer to other parts of the body.

**Radiotherapy**: in general, it is used to treat the rectum cancer and may be used either before or after surgery.
3) Methodology

In section 3.1 we describe how the samples of the three cell lines and the colonic tissues were prepared until they were seeded on the ZnSe crystals. In sections 3.2 and 3.3, we describe our spectrometer’s specifications, and how we chose our parameters. Band fitting, cluster analysis, LDA and ANN are the mathematical methods that we used to analyze our data, a brief description of these methods is given in section 3.4.

3.1 Sample Preparation

In this section we discussed the ways that we used in order to prepare our samples until we seeded them on zinc selenide crystals.

3.1.1 Cell lines and primary cells

3.1.1.1 H-ras murine fibroblasts

Balb/3T3 murine fibroblasts cell lines were transfected with the 6.6 Kbp BamHI fragment containing H-Ras oncogene from human T24 bladder carcinoma, cloned into the BamHI site of selected plasmid pSV2neo. These cell lines are grown as monolayers that are influenced from contact inhibition. The resultant clone 98/6 showed a high tumorigenicity in Balb/c mice, and transformed morphology in vitro, and is designated as “malignant”. Control cells 98/1 that were transfected only with vector plasmid were not tumorigenic and showed normal morphology in vitro, and are designated as “normal” [86]. The two cell lines were obtained from Dr. J. Gopas, Department of pathology, Soroka Medical Center, Ben Gurion University and Dr. N. Grossman, Department of Microbiology & Immunology, Faculty of Health Sciences, Ben-Gurion University and Soroka University Medical Center, Beer-Sheva.

3.1.1.1.1 The growing medium

The cells were grown in Dulbecco’s Modified Eagle Medium (DMEM). This medium contains glucose, salts vitamins and amino acids. It is also rich in Glutamine 2mM and different types of antibiotics to prevent the growth of undesired bacteria (Penicillin 100 U/ml, Streptomycin 100 µg/ml, Gentamycin 50 µg/ml) and 10% Fetal Calf Serum (FCS) [86].
3.1.1.1.2 Cell Growing.

After seeding the cells and after covering 80% of the growing plate it is necessary to dilute the cells. The dilution keeps the cells in the logarithmic growing phase. The medium should be pumped from the plate and the remaining medium should be washed in phosphate buffer saline (PBS). After that the PBS should be pumped and add trypsin. The trypsin is a digestive enzyme, which helps in removing the cells from the plate. The cells are collected to a centrifuge tube and a medium is added to the cells. After shaking the tube the cells were centrifuged for 3 minutes at speed of 1000 rpm. The upper layer of the fluid in the tube was pumped and the sediment was added to 30 ml medium. 17 ml were taken to a count cells and the number of cells per ml was counted. The rest of the cells were spread into a new growing plate and the plate was entered into the incubator 37°C, 8% CO₂ and 95% humidity.

3.1.1.1.3 Sample Preparation

Cells were seeded directly on 2x2 cm² ZnSe crystals at concentration of 1 million cells/slide, and after ensuring complete dryness, the FTIR measurements were made.

3.1.1.2 NIH and MuSV

3.1.1.2.1 Cells and Viruses

NIH/3T3 cells (mouse fibroblast cells) were grown at 37°C in RPMI medium supplemented with 10% new born calf serum (NBCS) and the antibiotics penicillin, streptomycin and neomycin.

Clone 124 of TB cells chronically releasing Moloney murine sarcoma virus (MuSV-124)(20) was used to prepare the appropriate virus stock. MuSV-124 stock contained an approximately 30-fold excess of MuSV particles over Moloney murine leukemia virus (MuLV) particles. MuSV used in this research was grown on NIH/3T3 cells. The virus concentration was determined by counting the number of foci (ffu-focus forming units).

3.1.1.2.2 Cell infection and determination of viral infection.

A monolayer of NIH/3T3 cells was grown in 9 cm² tissue culture plates and treated with 0.8 μg/ml of polybrene (a cationic polymer required for neutralizing the
negative charge of the cell membrane) for 24 h before infection with the virus. Free polybrene was then removed, and the cells were incubated at 37°C for 2 h with the infecting virus (MuSV-124) at various concentrations in RPMI medium containing 2% of NBCS. The unabsorbed virus particles were removed, and fresh medium containing 2% of NBCS was added, and the monolayers were incubated at 37°C. After 2-3 days, the cell cultures were examined for the appearance of malignant transformed cells. The amount of malignant transformed cells was expressed as the percentage of transformed cells in the inspection field or as the number of foci in the infected culture 10 days after infection [87].

3.1.1.2.3 Sample Preparation

Cells were seeded directly on ZnSe crystals. The samples were placed in the laminar flow for half an hour to ensure complete dryness, before taking the measurements.

3.1.1.3 Bone Marrow and MuSV

3.1.1.3.1 Cells and viruses

Primary rabbit cells obtained from the bone marrow of 1.5-kg rabbits were grown at 37°C in RPMI medium supplemented with 10% of newborn calf serum (NBCS) and the antibiotics penicillin, streptomycin and neomycin. Clone 124 of TB cells chronically releasing Moloney MuSV-124 was used to prepare the appropriate virus stock. The normal rabbit bone marrow fibroblasts were designated as BM cells, and those transformed by MuSV as BMT cells [88].

Dr. M. Huleihel has provided this cell line to us from, The Institute for Applied Biosciences, Ben-Gurion University of the Negev, Beer-Sheva, Israel

3.1.1.3.2 Cell proliferation

Cells, seeded at a concentration of 0.6 x 10^6 cells per 9.6 cm^2 plate, were incubated at 37°C in RPMI medium supplemented with 10% of NBCS and antibiotics. Each day, the cells were examined for morphological changes, and the number of cells was counted with a hemacytometer.

3.1.1.3.3 Cell infection and determination of cell transformation

A monolayer of Primary fibroblast rabbit grown in 9-cm^2 tissue culture plates was treated with 8 µg/ml of polybrene (a cationic polymer required for neutralizing
the negative charge of the cell membrane) for 24 h before infection with the virus. Excess polybrene was then removed, and the cells were incubated at 37°C for 2 h with the infecting virus (MuSV-124) at various concentrations in RPMI medium containing 2% of NBCS. The unabsorbed virus particles were removed, fresh medium containing 2% NBCS was added, and the monolayers were incubated at 37°C. After 2-3 days, the cell cultures were examined for the appearance of malignant transformed cells [88].

3.1.1.3.4 Sample preparation

Cells were grown to the required high densities on zinc selenide crystals, then washed twice with phosphate-buffered saline (PBS), dried, and examined by FTIR microspectroscopy. The same procedure was used for both the normal and the transfected cells.

3.1.2 Colonic tissues

Selected biopsy tissues obtained from patients are cut into small pieces and then fixed in 4% formalin solution for 12 hours. Thereafter the tissue was immersed in increasing grades of alcohol (70%-90%-95%-100%) 1 hour each, xylol for 3 hours and paraffin 3 hours. The fixed tissue is transferred into a mold were the tissue is embedded in paraffin and a block of paraffin is made. The blocks of paraffin are labeled and stored in an archive until required.

For FTIR measurements performed in this work, formalin-fixed, paraffin-embedded tissue from adenocarcinoma patients, were retrieved from the histopathology files of Soroka University Medical Center, Beer Sheva (SUMC). The tissue samples used in this study were selected to include normal, polyp and malignant areas. Two paraffin sections were cut from each case, one was placed on zinc-selenium slide and the other on glass slide. The first slide was deparaffinized using xylol and alcohol and was used for FTIR measurements. The second slide was stained with hematoxylin and eosin for normal histology review.

3.2 Why IR Microspectroscopy?

The colonic tissues contain different types of cells, epithel, blood, muscle, and lymphocyte cells. If we use the ordinary FTIR spectroscopy (using the
spectrometer compartment), then the spectrum will contain the characteristics of all these kinds of cells. If we compare the normal with the cancerous, it is therefore difficult to determine if the changes are due to differences in epithelial cells or as a result of the fact that different kinds of cells were measured. Therefore this study was made possible only using the recently developed FTIR microspectroscopy system. This development made such medical application of FTIR possible. Figure 3.2.1 shows the absorption spectra for different kind of cells observed on a single biopsy sample of colon tissue. The major difference is in the symmetric phosphate region in the range 1000-1150 cm\(^{-1}\). The peak is very clear in the epithel and lymphocytes spectra, while it is very weak in the case of muscle and blood spectra. Red blood cells do not contain nuclei therefore; they do not have RNA or DNA content. This is maybe the reason for the disappearance of the phosphate peaks. Muscle cells also contain small phosphate peaks indicated that DNA & RNA contribution is minor relative to epithel and lymphocytes [7]. The exact reason for this phenomenon is still unclear. There is a peak in the spectrum of red blood cells at 1160 cm\(^{-1}\).

Figure 3.2.1: Absorption spectra of different types of cells observed in colonic tissues. [The spectra were shifted vertically arbitrarily to give better view of the spectra.]

3.3 FTIR Microspectroscopy

FTIR-measurements were performed in transmission mode. For the transmission measurement, we used the FTIR microscope (IRscopeII) with liquid nitrogen cooled MCT detector, which is coupled to the FTIR spectrometer (BRUKER EQUINOX model 55 OPUS software) Figure 3.3.1.
Figure 4.3.1 a: View of the IRscope II microscope and the Bruker FTIR Spectrometer (Bruker Equinox 55) at BGU.

Figure 4.3.1 b: Schematic view of the infrared microscope spectrometer connected to the FTIR spectrometer at BGU (Bruker EQUINOX 55).

IS- Infrared source; MI- Michelson interferometer; A- Aperture; C1 and C2 Condenser and Objective respectively; S- Sample on computer-controlled microscope stage; M- Moveable mirror; MCT-IR detector; Visual camera and eye piece.
3.3.1 EQUINOX 55 Spectrometer

The interferometer is controlled using a HeNe – laser. The laser beam makes interference when passing through the interferometer, measuring the destructive interference points of the laser enable us to track the position of the moving mirror in accuracy of half $\lambda$. The laser emits 632.8 nm (red) light. The laser output is 1 mW.

3.3.1.1 EQUINOX 55 Specifications

The EQUINOX 55 optical system base configuration/specification is shown in table 3.3.1 [89].

**Table 3.3.1: EQUINOX 55 optical system base configuration/specification**

<table>
<thead>
<tr>
<th>Feature</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight</td>
<td>40 kg</td>
</tr>
<tr>
<td>Frequency Range</td>
<td>7500 – 370 cm$^{-1}$</td>
</tr>
<tr>
<td>Resolution</td>
<td>Better than 0.5 cm$^{-1}$ (Apodized)</td>
</tr>
<tr>
<td>Construction</td>
<td>Sealed optics housing, Desiccated, or purged.</td>
</tr>
<tr>
<td>Source</td>
<td>Air cooled (user replaceable)</td>
</tr>
<tr>
<td>Beamsplitter</td>
<td>Multilayer coating on KBr</td>
</tr>
<tr>
<td>Detector</td>
<td>DLATGS with KBr window</td>
</tr>
<tr>
<td>Sample compartment</td>
<td>25.5 x 26 x 19 cm, sealed from optics by KBr windows, purgable.</td>
</tr>
<tr>
<td>Interferometer</td>
<td>Mechanical interferometer with ROCKSOLID™ alignment (no compressed air required)</td>
</tr>
<tr>
<td>Scanner</td>
<td>Four computer selectable mirror velocities scan rate of 8 spectra/sec (8 cm$^{-1}$ resolution)</td>
</tr>
<tr>
<td>Electronics</td>
<td>Microprocessor controlled optics bench with digital speed control, system diagnostics, advanced system check, 16 bit-100 kHz A/D converter with 19 bit dynamic range, acquisition processor (AQP) with 4 Megabytes of RAM.</td>
</tr>
</tbody>
</table>

Figure 3.3.2 shows the Spectrometer – Inside View. The interferometer compartment components are shown in table 3.3.2.
The interferometer compartment components are shown in table 3.3.2.

**Table 3.3.2:** The interferometer compartment components.

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Sample Compartment</td>
</tr>
<tr>
<td>B</td>
<td>Interferometer Compartment</td>
</tr>
<tr>
<td>C</td>
<td>Source Housing</td>
</tr>
<tr>
<td>D</td>
<td>Power Supply Compartment</td>
</tr>
<tr>
<td>E</td>
<td>Detector Compartment</td>
</tr>
</tbody>
</table>

The EQUINOX 55 is equipped with a single IR source as shown in Figure 3.3.3. This high-efficiency IR source has a powerful collecting mirror with an optical feedback to guarantee source output stability.

**Figure 3.3.3:** Source Housing- Side View (cross section-1), Cut Away Rear View (2) [89].
3.3.2 IRscope II

The microscope is also equipped with a CCD-camera for the visible range of the spectrum. Since the ordinary glass slides have strong absorption in the wavelength range of our interest, we used Zinc Selenide crystals, which are highly transparent to IR light. Major factors in selecting a window material of Zinc Selenide are, transmission range, and reactivity with the sample and insolubility in water so it can be cleaned easily and reused. Table 3.3.2.1 gives the properties of infrared windows material [18].

Table 3.3.2.1: properties of infrared windows material

<table>
<thead>
<tr>
<th>Window Materials</th>
<th>Transmission Range (cm⁻¹)</th>
<th>Solubility in Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium chloride, NaCl</td>
<td>40000-625</td>
<td>Soluble (strong)</td>
</tr>
<tr>
<td>Potassium bromide, KBr</td>
<td>40000-385</td>
<td>Soluble (strong)</td>
</tr>
<tr>
<td>Potassium chloride, KCl</td>
<td>40000-500</td>
<td>Soluble (strong)</td>
</tr>
<tr>
<td>Cesium iodide, CsI</td>
<td>33000-200</td>
<td>Soluble (very strong)</td>
</tr>
<tr>
<td>Fused silica, SiO₂</td>
<td>50000-2500</td>
<td>Insoluble</td>
</tr>
<tr>
<td>Calcium fluoride, CaF₂</td>
<td>50000-1100</td>
<td>Soluble (weak)</td>
</tr>
<tr>
<td>Barium fluoride, BaF₂</td>
<td>50000-770</td>
<td>Soluble (weak)</td>
</tr>
<tr>
<td>Thallium bromide-iodide, KRS-5</td>
<td>16600-250</td>
<td>Soluble (weak)</td>
</tr>
<tr>
<td>Silver bromide, AgBr</td>
<td>20000-285</td>
<td>Soluble (very weak)</td>
</tr>
<tr>
<td>Zinc sulfide, ZnS (Irtran-2)</td>
<td>10000-715</td>
<td>Insoluble</td>
</tr>
<tr>
<td>Zinc Selenide, ZnSe (Irtran-4)</td>
<td>10000-515</td>
<td>Insoluble</td>
</tr>
<tr>
<td>Polyethylene (high density)</td>
<td>625-30</td>
<td>Insoluble</td>
</tr>
</tbody>
</table>

3.3.2.1 General:

The IRscope II has three modes of operation, Vis, IR and Vis/IR. By pressing a button we can switch between these modes of operation. One of the important advantages of the IRscope II as an optical microscope to see the sample area through a binocular eyepiece. We inspected the sample, then, we switched the IR mode and gathered the spectra from the same site. By selecting IR/VIS mode, it is possible to
measure the spectra along with the visible inspection. By pressing a button, it is possible to switch measurements from transmission mode to reflection mode.

3.3.2.2 Stages

A manual or automatic controls are available, this stage allows the user to change the sample position with fine displacements.

Table 3.3.2.2 shows the IRscope II specifications used in the present work.

Table 3.3.2.2: IRscope II specifications.

<table>
<thead>
<tr>
<th>Specification</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spectral Range</td>
<td>600 to 7000 cm(^{-1})</td>
</tr>
<tr>
<td>Spectral Resolution</td>
<td>Spectroscopic resolution (dependent on the FTIR spectrometer). In our study 4 cm(^{-1}).</td>
</tr>
<tr>
<td>Adjustment Accuracy</td>
<td>±1 µm in the measured spot.</td>
</tr>
<tr>
<td>Visual Field</td>
<td>Ca. 900 µm diameter (15x objective).</td>
</tr>
<tr>
<td>Working Distance</td>
<td>24 mm (with standard objective).</td>
</tr>
</tbody>
</table>

3.3.2.3 Space

The IRscope II is 36 cm wide x 58 cm deep and 56 cm high without video camera attached.

For optimum performance, a free dust, moisture and temperature controlled environment that is free of corrosive vapors is essential. Temperature limits are 18-30\(^\circ\) C and less than 70% humidity.

3.3.2.4 Optical Specifications

The IRscope II optical specifications are given in table 3.3.2.3.

Table 3.3.2.3: IRscope II optical specifications

<table>
<thead>
<tr>
<th>Objective</th>
<th>Objective Magnification</th>
<th>Visible magnification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard IR Objective</td>
<td>15x</td>
<td>150x</td>
</tr>
<tr>
<td>Visual objective</td>
<td>4x</td>
<td>40x</td>
</tr>
</tbody>
</table>
Figure 3.3.4 shows the optical path of IRscope II transmission mode.

In order to get the best spectral quality, we have to measure the background with the same aperture size as used for the actual sample measurement. The background spectrum must be measured with a clean portion of the supporting material when the sample is put on it. One of the reasons of low quality spectrum is partially due to bad background measurement. The optical parameters that we used in our microscopy measurements are shown in table 3.3.2.4.

Figure 3.3.4: Optical path-transmission mode used in the present study [89].
Table 3.3.2.4: Optical parameters used in our measurements.

<table>
<thead>
<tr>
<th>Optic Parameter</th>
<th>Situation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optical filter setting</td>
<td>Open</td>
</tr>
<tr>
<td>Aperture Setting</td>
<td>1.4 mm. (this parameter is determined in the software).</td>
</tr>
<tr>
<td>Measurement Channel</td>
<td>Microscopy</td>
</tr>
<tr>
<td>Detector setting</td>
<td>Microscope MCT</td>
</tr>
<tr>
<td>Scanner velocity</td>
<td>20 KHz</td>
</tr>
</tbody>
</table>

Acquisition Parameters that we used in our microscopy measurements are shown in Table 3.3.2.5.

Table 3.3.2.5: Acquisition Parameters used in our measurements.

<table>
<thead>
<tr>
<th>Acquisition Parameter</th>
<th>Situation</th>
</tr>
</thead>
<tbody>
<tr>
<td>High Frequency Limit</td>
<td>4000 cm(^{-1})</td>
</tr>
<tr>
<td>Low Frequency Limit</td>
<td>600</td>
</tr>
<tr>
<td>Acquisition mode</td>
<td>Double sided, Forward Background</td>
</tr>
<tr>
<td>Apodization Function</td>
<td>Blackman-Harris 4-Term</td>
</tr>
<tr>
<td>Zerofilling</td>
<td>2</td>
</tr>
<tr>
<td>Resolution</td>
<td>4 cm(^{-1})</td>
</tr>
<tr>
<td>Number of Scan</td>
<td>At least 128</td>
</tr>
<tr>
<td>Measured area (circular diameter)</td>
<td>50-100 (\mu m)</td>
</tr>
</tbody>
</table>

3.4 Data Acquisition

While measuring the colonic tissues, the pathologist examined the histology of each site using additional combined optical microscopy under standard histological conditions after staining. The measured areas of samples were typically a 100 \(\mu m\) diameter. The measured spectra cover the wavenumber range 600-4000 cm\(^{-1}\) in the mid-IR region. The spectrum was taken as an average of 128 scans to increase the signal to noise ratio. The spectral resolution was set at 4 cm\(^{-1}\). The baseline was corrected using Rubberband correction. We used two methods to normalize our spectra, min-max normalization and vector normalization.
In the FTIR measurements of cell lines and the bone marrow primary cells, a biologist examined on-line the optimum sites for measurement.

Each measurement day, the container of the MCT detector was filled with liquid nitrogen about 30 min before measurements, and a self-test of the instruments was done. This test included alignment, and calibration according to standard polystyrene foil positioned inside the Bruker FTIR spectrometer.

3.5 Advanced Computational Methods

Our main goal in this study is to differentiate between normal and cancerous cells in the cell line and among normal, polyp and cancerous colonic tissues, to find the biological markers and to understand the difference in the cell metabolites. It was difficult to differentiate between polyp and cancerous colonic tissues using FTIR spectra and the biological markers. In order to get good classification between polyp and malignant samples, we investigated the use of advanced computational methods. Artificial Neural Network (ANN) gives better classification for polyp and malignant types than the other computational methods such as Cluster analysis and LDA.

3.5.1 Spectral Analysis and Band Fitting

In this work we used biological samples, which are rich in vibrational modes. Due to the overlapping of the absorption peaks, there is more than one vibration mode in some peaks, which appear as doublets or triplets. In order to locate these hidden peaks, we used the PeakFit version 4. The program allows to make band fitting and to detect the hidden peaks. PeakFit calculates the second derivative of the spectrum to locate the peak centroids [90]. The number of peaks was chosen according to the features of the original spectrum [91].

The spectra were fitted using a standard Gaussian peak shape:

\[ I(\nu) = I_0 \exp\left[ -\frac{1}{2}\left(\frac{\nu - \nu_0}{w}\right)^2 \right] \]

where \( I(\nu) \) represents the IR absorbance at wavenumber \( \nu \), \( \nu_0 \) is the peak centroid and \( w \) is the width parameter. The full width at half maximum (FWHM) of the band \( \Gamma \) is calculated using the relation \( \Gamma = 2.36 \ w \). The background was fitted using a linear function. Weak absorption bands can be resolved by using modern FTIR spectrometers. The centroids, widths and absorption amplitudes were varied.
simultaneously to minimize $\chi^2$ for the entire fit. Using the program, it is possible to fit a chosen number of bands simultaneously. All parameters have been kept free. The spectra of normal, polyp and cancerous tissue samples were fitted separately. Our findings show that many shoulders demanded fitting as two or more overlapping peaks. We used the second derivative method to detect the exact location of the hidden peaks, and the residual method to calculate the difference between the spectral fits and the actual data.

In addition, Lorentzian peak shape was also tested but the Gaussian peak shape gave better overall fit.

### 3.5.2 Cluster Analysis

Clustering techniques [92, 93] were used in our study to differentiate cancerous and normal samples and locate the most sensitive regions of the IR spectra for this purpose. Clustering techniques were used in other fields, like medicine, clustering diseases, cures for diseases, or symptoms of diseases, these techniques can lead to very useful taxonomies.

Cluster analysis [92, 93] is an unsupervised technique examining the interpoint distances between all the spectra. It shows the data in a two-dimensional plot (name of file vs. heterogeneity), referred to as dendrogram [92, 93]. Presenting data from high-dimensional row spaces, the dendrogram makes it possible for us to use our pattern recognition abilities. This method uses the distance between spectra in order to cluster them according to their nearness in the row space. Usually each spectrum is treated as a cluster in the beginning, and then the closest clusters are joined together. Figure 4.1.13 shows an example of a dendrogram of 20 samples of cancerous and their corresponding controls (BM, BMT cells), studied in the present work.

There are many methods to calculate the distances between clusters. The Ward method is an outstanding method since it employs an analysis of variance approach to evaluate the distances between clusters, it is considered very efficient. It tries to reduce the Sum of Squares of any two clusters to the minimum at each step [92].
3.5.3 Linear Discriminant Analysis (LDA)

LDA is a classification technique that employs Mahalanobis distance to determine the class of an unknown sample [94, 92]. In our study we wanted to compare cancerous with normal colonic spectra. The comparison is achieved by calculating the mean difference and standard differences of the two samples. This comparison is easy if one variable such as phosphate contents is calculated in each measurement. Since there is overlapping between the normal and cancerous groups using one metabolite only, we used LDA method, which is a generalization of the notation of standard distance that allows to use all variables simultaneously (such as phosphate level, RNA/DNA ratio, carbohydrate level) in order to compare the two samples. There are correlations between the variables, thus the test statistics computed for each variable individually is not independent. It turns out that this generalization is closely related to a generalization of the well-known two-sample \( t \)-statistics and to multiple regression model [94, 92, 95, 96].

To fully evaluate the performance of the proposed method, linear discriminant analysis (LDA) [97] was employed using MATLAB (Version 5.3: Math Works Inc.). In this study, training and test sets were selected randomly from the database. Fifty percent of each set was employed for training and the remainder for test. In addition, the validation experiment was repeated 100 times, with the same input features but with different sets of randomly selected training and test sets, and the results were averaged.

3.5.4 Artificial Neural networks (ANNs)

3.5.4.1 What is a Neural Network

Artificial neural networks (ANNs), which are inspired by biological neural systems, emulate a part of the observed characteristics of the biological nervous system in mathematical models. These models are information processing systems that can learn, such as adaptive biological learning, and help us solve many problems. The ANN paradigm is characterized by a novel structure of information processing system. Like biological neurons, it consists of large number interconnected processing elements. These elements are linked with weighted connections similar to synapses (Figure 3.5.4.1). Figure 3.5.4.1 shows a simple neuron cell. The neuron consists of dendrites (nerve fiber), the cell body of the soma,
and an axon. The dendrites are connected to the cell body or soma, where the cell nucleus is located. The axon branches into strands and substrands that are connected to other neurons through synaptic junctions, or synapses.

![Neuron Diagram]

**Figure 3.5.1:** A simple neuron cell [38].

In order to configure an ANN for a specific application, such as pattern recognition or data classification, the ANN should pass a learning process, which can be achieved by learning through examples. Similar to learning in biological systems, learning in ANN system is achieved by changing the connection weights.

### 3.5.4.2 Historical Background

Warren McCulloch, neurophysiologist, and Walter Pits, a logician, invented the first artificial neuron in 1943 [98], but because of the undeveloped technology in their time, they could not proceed.

As a good pattern recognition engines and robust classifiers, ANNs are now used in many fields to solve real and complex problems in chemistry, biology, pathology and medicine [99, 100, 101, 102, 103, 104]. ANNs make it possible to make generalizations about imprecise input data. Detailed account of ANN can be obtained from the excellent books [105, 106], and review article [107].

### 3.5.4.3 Advantages of Neural Network

ANNs are resilient against distortions in the input data, and they also have the following advantages [108, 109]:

1. **Adaptive learning**: learning how to perform tasks depending on initial experience or the data received for training.

2. **Self-Organization**: ANN organizes and represents the information received while learning.

3. **Real Time Operation**: ANN computations may be performed in parallel. Moreover, it is possible to take advantage of this capability by means of the special designed and manufactured hardware.

### 3.5.4.4 Building A Neural Network

The pattern recognition device with learning capabilities, called the “Perceptron”, was proposed by the psychologist Frank Rosenblatt in 1958 [110]. Since then, the most widely studied form of network structure is this hierarchical neural network. A hierarchical neural network links multiple neurons together hierarchically, as shown in Figure 3.5.4.2.

![Figure 3.5.2: A feed forward neural network.](image)

Simple dynamics is a major quality of this network. A signal that gets into the input layer passes to the next layer by the neuron interconnections. The receiving layer’s neurons process the signal before moving it to the next layer. The complete signal processing ends when the signal reaches the output layer.

The signal transformation depends on the way that the hidden layers process the input signal. The weight and threshold parameters in each unit of the hierarchical network influence its dynamics. Effectively using hierarchical networks to process information in conditioned by adjusting parameter values in order to get proper outputs of the input signals.

It is a difficult task to determine multiple parameter values precisely. Consequently, a learning method, involving a network that randomly determines parameters values, is needed to perform input-to-output transformations of real
problems. The proper modification of parameters according to errors of the network determines the correct final parameters as shown in Figure 3.5.4.3.

By changing the weights slightly according to the chosen learning algorithm, the learning capability of the artificial neuron is achieved. The error back-propagation learning method proposed by D. E. Rumelhart et al. in 1986, which has played a major role in the recent neurocomputing boom, is the best representative one of the few methods that have been proposed [111].

![Figure 3.5.3: Supervised network with back propagation learning rule.](image)

### 3.5.4.5 Multilayer Perceptron

Multilayer Perceptron (MLP), which is generally trained with the back propagation of error algorithm, is the most popular type of ANNs. MLP has been commonly used in many NN applications due to its simple implementation. The network consists of many processing elements connected in several layers. The output of one processing element is connected to the input paths of other processing elements through connection weights. When presented repetitively with the input and the desired output the MLP organizes internally, gradually adjusting the weights to achieve the desired input/output mapping. Given enough data it is possible to design and teach a MLP with one hidden layer to reproduce the behavior of any process linear or non-linear. The training process is showed in Figure 3.5.4.3, the network is
trained or “taught” by showing a set of examples, observing the network answers and correcting the “wrong” answers by changing or adapting the network weights.

### 3.5.5 Multiscale Decomposition by Fast Wavelet Transform (FWT)

This analysis has been used and explained in our paper [112]. We give here only a brief description of FWT method. Further details can be found in ref [113]. We performed a multiscale decomposition of the spectrum to examine the performance of the NN based classifier [112]. The wavelet transform is an important tool for signal analysis and feature extraction [113]. It gives a good local representation of the signal in both the time domain and the frequency domain. Figure 3.5.6.1 a & b shows that the Fourier transform (FT) is global and describes the overall regularity of the signals, and that the wavelet transform looks for the spatial distribution of singularities. Both FT and the FWT decompose the signal but differently; the FT decomposes it into a series of sinusoidal components, and the FWT decomposes it into a series of wavelets of different scales and positions.

We used the FWT proposed by Mallat and Zhong [113] in this work. We decomposed the spectrum into an orthogonal set of waveforms that are the dilations, translations and modulations of the Coiflet wavelet (mother wavelet). We chose the Coiflet wavelet because its results were the best in comparison with other wavelets. We convolved the spectrum with these dilated wavelets and computed the wavelet transform.

The wavelet’s coefficients of the different scales offer a compact representation of the spectrum signal. It is evident that the transform involves differentiation and progressive smoothing. Details of the peaks are gradually lost as the downward slopes of the wave are being picked up at higher scales. The number of scales is to be chosen by searching for the optimal signal representation.
4) Results

4.1 Cell lines and Primary cells:

In this study we used two different cell lines and primary cells listed below:

1. Balb/c murine fibroblasts were transfected by H-ras oncogene from the human T24 bladder carcinoma [44] clone 98/6. Control cells (98/1) were not tumorigenic and showed normal morphology. In the following we use the label f1 for 98/1 clone and f6 for 98/6 clone.
2. NIH/3T3 cells (mouse fibroblast cells) were transfected by MuSV. Clone 124 of TB cells chronically releasing Moloney murine sarcoma virus (MuSV-124) [45] was used to prepare the appropriate virus stock.

3. The cell cultures were examined for the appearance of malignant transformed cells.

4. Primary rabbit cells obtained from the bone marrow of 1.5-kg rabbits were transfected by MuSV. Clone 124 of TB cells chronically releasing Moloney MuSV-124 [45] was also used to prepare the appropriate virus stock. The cell cultures were examined for the appearance of malignant transformed cells.

Cell lines unlike the “real” tissues have advantages in spectroscopy techniques since they are homogeneous, and therefore the measurements are from one type cells. All the cells are either normal or malignant under controlled conditions. It is important to use these homogeneous samples in order to check the potential of the new method before using it in a very difficult and complicated sample such as real tissues. Also in our cell lines the cause of the cancer is known.

Primary cells are similar to the cells in tissues. They replicate slowly and they die after few passages. Therefore it was important to study in this work, both regular cell lines and primary cells. The cell lines used in this work are summarized in table 4.1.1.

Table 4.1.1: Description of cell lines and primary cells used in this work

<table>
<thead>
<tr>
<th>Name of cell lines/type</th>
<th>Description</th>
<th>Label in the work</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Balb/c</td>
<td>Murine fibroblast</td>
<td>f1 and f6</td>
</tr>
<tr>
<td>2 NIH/3T3</td>
<td>Mouse fibroblast</td>
<td>NIH and MuSV</td>
</tr>
<tr>
<td>3 Primary rabbit cells</td>
<td>Bone marrow fibroblast</td>
<td>BM and BMT</td>
</tr>
</tbody>
</table>

4.1.1 Pictures of cells

4.1.1.1 Normal and malignant cells f1 and f6

Photo 4.1.1a and Photo 4.1.1b show a picture of f1 and f6 cells respectively. These photos were taken using a ZEISS microscope (LSM 510) equipped with a
CCD camera in their growing dishes. There is a morphological change between the two types of the cells. The controls have circular shape and they are homogenous, while the tumorigenic cells look less homogenous and have sharp edges. Growth curves of the two cell lines were determined using proliferation parameters (number of cells) and are displayed in Figure 4.1.1.1. The growing rate of the cancerous cells is higher than their controls. The error bars were calculated as the standard deviation of the number of cells counted from different squares.

Photo 5.1.1: (a) Normal Balb/c 3T3 murine fibroblasts cells (f1). (b) Balb/c 3T3 cells transfected by H-ras (f6).
4.1.1.2 Normal and malignant cells BM and BMT

Primary rabbit BM fibroblasts grown in plastic dishes in RPMI medium with 10% NBCS appear as flat cells under an inverted light microscope (Photo 4.1.2a staining). These cells are completely unable to grow in soft agar. When BM cells were infected by MuSV, transformed cells with a highly refractive shape growing randomly in a criss-cross fashion were detected (Photo 4.1.2b). In contrast to BM cells, the BMT cells were able to grow on soft agar and to produce large colonies within 5-10 days. BM cells replicated very slowly in culture and could not survive high densities. Most of the BM died after about 10 passages. In contrast, the BMT cells grew rapidly and reached high densities in cell culture (Figure 4.1.1.2).
b)

Photo 4.1.2: (a) Normal primary rabbit fibroblast (BM) cells. (b) BM cells transformed by murine sarcoma virus (BMT).

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Figure 4.1.1.2: Cell proliferation of BM and BMT cells.
4.1.2 FTIR measurements of normal and malignant cells

Typical infrared absorption spectra of normal murine fibroblasts f1, normal mouse fibroblasts NIH and normal rabbit bone marrow fibroblasts BM in the range 600-4000 cm$^{-1}$ are shown in Figure 4.1.2.1. The spectrum is dominated by two absorbance bands at 1654 and 1544 cm$^{-1}$ known as amide I and II respectively. Amide I arises from the C=O hydrogen bonded stretching vibrations, and amide II from the C-N stretching and a CNH bending vibrations. The weaker protein bands at 1457 and 1399 cm$^{-1}$ are associated with the asymmetric and symmetric CH$_3$ bending vibrations. The peaks at 1241 and 1086 cm$^{-1}$ are attributed to PO$_2^-$ ionized asymmetric and symmetric stretching, respectively [114]. The bands at 3293 and 2924 cm$^{-1}$ are CH$_2$, CH$_3$ stretching vibrations of cholesterol and phospholipids respectively.

![Figure 4.1.2.1: Infrared microspectroscopy from three different sources of normal cell lines fibroblasts.](image)

FTIR spectra of normal fibroblasts (NIH/3T3) and malignant cells transformed by MuSV are presented in the Figure 4.1.2.2. From Figure 4.1.2.2, it can be seen that the absorption due to normal cells was significantly higher than that for malignant cells over the entire region of the spectrum. This trend is true also for
normal BM fibroblasts and of the retrovirally transformed cells [BMT] (Figure 4.1.2.3), and for normal f1 and H-ras transfected fibroblasts (Figure 4.1.2.4).

**Figure 4.1.2.2.** FTIR spectra of normal and malignant fibroblast cells transformed by murine sarcoma virus (MuSV).

**Figure 4.1.2.3:** Infrared spectra in the region 600-4000 cm\(^{-1}\) of normal (BM) and transformed (BMT) rabbit bone marrow fibroblasts.
Figure 4.1.2.4: Infrared microspectroscopy of normal and cancerous mouse fibroblasts in the range 600-4000 cm$^{-1}$.

Similar notable spectral differences between normal and ras transfected types were observed in all the cases studied after normalizing to Amide I.

FTIR spectra of normal (two samples) and H-ras transfected fibroblasts (two different batches) are displayed in the region 1000-1500 cm$^{-1}$ in Figure 4.1.2.5. Amide II showed no significant change in the intensity upon transformation. The regions 1000-1200 and 1400-1500 cm$^{-1}$ displayed marked changes in the intensity due to the expression of the H-ras oncogene. Also, no frequency shifts were observed in the entire region of the spectrum. Figure 4.1.2.6 shows microscopic FTIR spectra of normal and H-ras transfected fibroblasts for additional two different samples in the wavenumber range 600-1800 cm$^{-1}$. Also in these cases the magnitude of absorbance of the normal cells was higher than the transformed by H-ras oncogene mainly in the region 1000-1600 cm$^{-1}$.
Figure 4.1.2.5: Infrared microspectroscopy of two normal and H-ras transfected mouse fibroblasts. The spectra a, c and b, d are normal and H-ras transfected fibroblasts of two different batches respectively.

Figure 4.1.2.6: Infrared microspectroscopy of normal and cancerous murine fibroblasts in the range 600-1800 cm\(^{-1}\).

FTIR spectra of normal fibroblasts (NIH/3T3) and malignant cells transformed by MuSV are presented in the Figure 4.1.2.7 in the region 600-1800 cm\(^{-1}\).
Figure 4.1.2.7: FTIR spectra of normal and malignant fibroblast cells transformed by murine sarcoma virus (MuSV).

Figure 4.1.2.8 represents the FTIR-MSP spectra of normal (BM) and transformed (BMT) rabbit bone marrow fibroblasts. The intensities of the spectra of normal BM fibroblasts were significantly higher than those of the retrovirally transformed cells. The most significant differences in the spectral intensities were evident in the 1000-1630 cm\(^{-1}\) region. The full width at half maximum (FWHM) of amide I peak was larger for normal than for malignant cells. No major spectral pattern changes were observed between the two types of cells.

To understand the effect of various methods of spectral processing, vector normalized FTIR-MSP spectra of normal (BM) and transformed (BMT) cells are shown in Figure 4.1.2.9.

The vector normalized spectra also showed that the absorbance for normal (BM) cells was higher than the transformed (BMT) except for amide I band where absorbance changes were reversed. Hence it can be concluded that the quantitative changes observed in our study in the fingerprints regions (e.g., phosphate) were independent of methods of spectral normalization.
Figure 4.1.2.8: Infrared spectra in the region 600-1800 cm\(^{-1}\) of normal (BM) and malignant (BMT) fibroblast cells transformed by murine sarcoma virus (MuSV).

For comparison, the absorbance changes between normal (NIH/3T3) and transformed (NIH/3T3-MuSV) cells were similar to the progenitor cells isolated from bone marrow. The percentage change in absorbance between these two different sources can be better understood from the difference spectra shown in Figure 4.1.2.10. Subtracting the BMT from the BM spectrum gives the spectrum (labeled as blue) (BM – BMT), while the red line was generated by subtracting the NIH_MuSV from the NIH/3T3 spectrum (NIH/3T3 – NIH_MuSV). The percentage change was much higher for the difference spectra BM-BMT than the NIH-MuSV indicating that FTIR-MSP might be specific in identifying the retroviral infection of fibroblasts obtained from different sources.

4.1.3 Spectral Analysis and Band Fitting

Spectral analysis and band fitting was performed in the region 600-1730 cm\(^{-1}\) in the present work and is shown in Figure 4.1.3.a & b. The exact peak location of the bands and the "hidden" peaks, which appear on the shoulder of major peaks were extracted using the second derivative spectra presented in Figure 4.1.3b. Figure 4.1.3a shows a typical case for normal (f1) fibroblast cells. Figure 4.1.3c shows the residue of the above spectral fit.
Figure 4.1.2.9: Vector normalized FTIR- microscopic spectra of controls and MuSV transformed fibroblasts isolated from bone marrow of rabbits.

Figure 4.1.2.10: FTIR difference spectra generated by subtraction of the transformed cells spectrum from the normal cells spectrum for two cell lines NIH/3T3 and rabbit bone marrow fibroblasts.
Similar residue spectra (not shown) were obtained for cancerous cells. The same fitting procedure was used for all the normal and malignant fibroblast samples. As can be seen, each minimum in the second derivative spectrum is contributed by a single peak in the original spectrum.

### 4.1.4 Phosphate Levels

Quantification of phosphate metabolites composed of energy yielding molecules and nucleic acids provide a clue about the various states of the cell [115]. The analytical area calculated for peaks 13 and 17 (located at 1086 and 1241 cm\(^{-1}\), which arise from the symmetric and asymmetric phosphate stretching vibrations respectively) for normal (f1) and cancerous (f6) cells respectively are shown in Figure 4.1.4.1. The error bars were calculated as following: 1. We measured more than five spectra from each sample. 2. The measured spectra were averaged to give a representative spectrum of the sample. 3. The error bars shown in the spectrum represent the standard errors of phosphate levels for each individual measurement. The results indicate that the absorbance bands for phosphate levels were higher in normal (f1) cells than the ras transfected cells in all five cases studied in this work.

For the systems NIH/3T3 and NIH-MuSV, and BM and BMT fibroblast cells, the phosphate level was determined as the sum of the integrated intensities of phosphate symmetric vibration in the range 980-1149 cm\(^{-1}\) and phosphate asymmetric vibration in the range 1151-1350 cm\(^{-1}\) using OPUS software. The areas under the phosphate absorption peaks are shown in Figure 4.1.4.2 and Figure 4.1.4.3 for NIH/3T3 and NIH-MuSV, and BM and BMT respectively. These latter Figures give a clear picture of the difference in phosphate contents between normal and malignant cells, in all the tested samples, the phosphate content of normal cells was significantly higher than that of the malignant cells. The variation among the transformed (BMT) cells was lower compared to the controls.

### 4.1.5 RNA/DNA ratio

Progress in malignant transformation of the cell can be deduced from RNA/DNA, which could be one of the biological markers. Earlier reports [116] indicated that malignant cells had increased RNA/DNA compared to the controls. To test this hypothesis in the present model, the 1121/1020 ratio was calculated for
Figure 4.1.3: (a) Typical band fitting analysis in the wavenumber region 1730-600 cm$^{-1}$ for sample number 5 (normal cells, f1). The dots represent the data points and the blue solid line represents the overall fit. (b) Second derivative spectrum used for the assignments of exact peak centroids. (c) The residue spectrum was calculated as the difference between the fitted and the original spectrum.
Figure 4.1.4.1: Summed analytic areas of the two phosphate bands labeled 13 (symmetric) and 17 (asymmetric) shown in Figure 4.1.3 (a).

Figure 4.1.4.2: Integrated areas for peaks at 1241 cm$^{-1}$ and 1086 cm$^{-1}$ represent phosphate levels for all normal and malignant samples.
Figure 4.1.4.3: Phosphate level as a biological marker derived from the FTIR spectra for controls and MuSV transformed fibroblasts isolated from bone marrow of rabbits.

Figure 4.1.5.1: RNA/DNA for all five normal and H-ras transfected fibroblasts calculated from the intensity ratio $I(1121)/I(1020)$.
Figure 4.1.5.2: Intensity ratio of 1121 cm$^{-1}$/1020 cm$^{-1}$ represents RNA/DNA for normal (NIH/3T3) and malignant (NIH-MuSV) cells.

Figure 4.1.5.3: Intensity ratio $I(1121)/I(1020)$, as representative of RNA/DNA, for all seven samples of normal (BM) and transformed (BMT). The results are displayed in Figures 4.1.5.1, Figure 4.1.5.2 and Figure 4.1.5.3. The results indicate that the RNA/DNA ratio was higher for cancerous cells than their corresponding controls.
### 4.1.6 FTIR microspectroscopy of normal and malignant cells in the higher wavenumber region

Cholesterol, phospholipids and creatine are three essential cellular metabolites, that absorb between 2800 and 3500 cm\(^{-1}\). Since there are symmetric and asymmetric vibrations attributed to water in the region between 3200-3550 cm\(^{-1}\), this region was not considered for detailed analysis.

Figure 4.1.6.1 displays the upper wavenumber region 2600-3500 cm\(^{-1}\) of the cell lines f1 and f6. The upper wavenumber region 2600-3500 cm\(^{-1}\) of the cell line NIH/3T3 and NIH-MuSV, is shown in Figure 4.1.6.2. The FTIR-MSP spectra of the primary cells (BM and BMT) in the region 2600-3500 cm\(^{-1}\) are presented in Figure 4.1.6.3. Here also, the magnitude of normal cells is higher than cancerous types in all our samples. The integrated areas for peak I and peak II for NIH and MuSV were calculated by extracting the area under the curve omitting the baseline underneath using OPUS software. Whereas the integrated areas for peak I and peak II for f1 and f6 were calculated by extracting the area under the curve without omitting the baseline underneath using OPUS software. The integrated areas calculated for the peaks at 2848 and 2916 cm\(^{-1}\) for all samples are displayed in Figure 4.1.6.4 (a & b) for f1 and f6 and Figure 4.1.6.5 (a & b) for NIH/3T3 and NIH-MuSV. The area for normal cells was higher than that of the cancerous fibroblast cells in all samples.

![Absorbance Wavenumber Graph](image)

**Figure 4.1.6.1:** Infrared microspectroscopy of normal and H-ras transfected mouse fibroblasts in the wavenumber region 2600-3500 cm\(^{-1}\).
Figure 4.1.6.2: Infrared microspectroscopy of normal (NIH/3T3) and transfected fibroblasts (NIH-MuSV) in the wavenumber region 2600-3500 cm\(^{-1}\).

Figure 4.1.6.3: Infrared microspectroscopy spectra in the region 2600-3500 cm\(^{-1}\) of BM and BMT.
Figure 4.1.6.4: Integrated area of two peaks: (a) peak I (2866 cm\(^{-1}\)) and (b) peak II (2930 cm\(^{-1}\)) for five samples.
Figure 4.1.6.5. (a) Integrated areas of peak I (2848 cm\(^{-1}\)) and (b) of peak II (2916 cm\(^{-1}\)) for all normal and malignant tested samples.
4.1.7 Carbohydrate Level

The bands at 1025 and 1045 cm\(^{-1}\) are attributed to the vibrational frequency of -CH\(_2\)OH groups and the C-O stretching frequencies coupled with C-O bending frequencies of the C-OH groups of carbohydrates (including glucose, fructose, glycogen, etc.) [117]. The ratio of the areas of the peak at 1045 cm\(^{-1}\) to that at 1545 cm\(^{-1}\) gives an estimate of the carbohydrate concentrations (Figure 4.1.7.1). Our results indicated that carbohydrate (including glycogen) levels were lower in malignant (MuSV) cells than in normal (NIH) cells. These results are in complete agreement with the decrease in phosphate levels in malignant (MuSV) cells shown in Figure 4.1.4.2.

The carbohydrate levels were also reduced in ras transfected cells (f6) in comparison with normal cells (f1) (Figure 4.1.7.2). This is in complete agreement with the decrease in phosphate levels presented in Figure 4.1.4.1.

![Carbohydrate Level Graph](image)

Figure 4.1.7.1. Intensity ratio of I(1045 cm\(^{-1}\))/I(1545 cm\(^{-1}\)) x 100, representing carbohydrate level for all normal and malignant tested samples.
Figure 4.1.7.2: Carbohydrate levels for all five samples, extracted from the intensity ratio at I(1045) / I(1545) X 100.

4.1.8 Ratio of amide I/II

Ratio of amide I/II bands was reported to shed light on the change in the DNA content [118]. The ratio of amide I/II is unity for RBCs and deviation from unity is an indication of DNA absorption from the cells. The contribution of the DNA due to carbonyl group from the bases is quantified by the ratio of integrated absorbance of amide I/II. Our results shown in Figure 4.1.8.1 indicated that transformed cells (BMT) had higher DNA content than the controls (BM). In 42% of the cases, the difference between controls and transformed cells was significantly high which was in accordance with the observations in the literature [118]. Also the amide I/II ratios were much higher for the malignant cells (NIH-MuSV) than for the normal cells (NIH/3T3) (Figure 4.1.8.2). The error bars were calculated as the standard errors of at least five measurements of the same sample.
Figure 4.1.8.1: The area of amide I (1654)/ II (1544) for controls and MuSV transformed fibroblasts isolated from bone marrow of rabbits.

Figure 4.1.8.2: Intensity ratio of 1654 cm\(^{-1}\)/1544 cm\(^{-1}\) calculated as the amide I/II ratio for all normal and malignant samples.
4.1.9 Glucose/Phosphate ratio

The ratio of the intensity at 1030 cm\(^{-1}\) to that at 1080 cm\(^{-1}\) gives an estimate of the Glucose/phosphate, which is a reliable measure for metabolic turnover of the cells and it is presented in Figure 4.1.9.1 for (f1 and f6) and Figure 4.1.9.2 for (NIH and MuSV). Predominantly, the normal cells gave rise to higher ratio than the cancerous cells and this is in good agreement with other metabolites such as DNA, protein and lipids.

4.1.10 Glucose/Phospholipids ratio

The glucose/phospholipids is the measure of de novo synthesis of phospholipids at the expense of the free glucose level in the cells. It is calculated as the ratio of the intensity at 2924 cm\(^{-1}\) to that at 1080 cm\(^{-1}\). Figure 4.1.10 shows the level of glucose/phospholipids for the samples studied. This ratio was found to be larger in the case of normal cells (f1) relative to the transformed by H-ras.

![Figure 4.1.9.1](image-url)  
**Figure 4.1.9.1:** The intensity ratio at 1030/1080 is shown as glucose/phosphate for normal (f1) and H-ras transfected (f6) cells.
Figure 4.1.9.2: Intensity ratio of 1030 cm\(^{-1}\)/1080 cm\(^{-1}\) represents glucose/phosphate ratio for normal (NIH/3T3) and malignant (NIH-MuSV) samples.

Figure 4.1.10: The intensity ratio at 1030/2924 is presented as glucose/phospholipids for normal (f1) and H-ras transfected (f6) fibroblast cells.
4.1.11 FTIR Microspectroscopy of purified DNA from normal (NIH/3 T3) and malignant (NIH-MuSV) cells

FTIR spectra were obtained for purified DNA from NIH/3T3 cells and from the corresponding malignant cells transformed by MuSV. The spectra were vector normalized. The centroid of the major peak at 1045 cm\(^{-1}\) is identical for both sources of DNA. This peak is due to ribose C—O and the PO\(_2\) group stretching vibration [119]. Our results (Figure 4.1.11) showed that the absorbance due to skeletal cis and trans conformations (CC) of DNA (absorbance bands at 1076 and 1035 cm\(^{-1}\)) was significantly higher in DNA from malignant cells than in DNA from normal cells.

![FTIR spectra of purified DNA](image)

**Figure 4.1.11:** FTIR spectra of purified DNA extracted from normal and malignant fibroblast cells transformed by MuSV.

4.1.12 2D Plots

Two-dimensional plots are good representation of the variation of the related metabolites. Figure 4.1.12.1 illustrates the variation of phosphate with carbohydrate levels in H-ras transfected and the controls. There was nearly a linear correlation found between these two metabolites in both types of the cells. The phosphate level was higher for normal cells along with its corresponding carbohydrate levels than the H-ras transfected malignant cells. The transformed cells varied to a larger extent in this plot than the normal cells.
Plot of RNA/DNA vs. glucose/phosphate (Figure 4.1.12.2) showed that there was a negative correlation between these two parameters. For majority of the H-ras transfected cells, RNA/DNA increased with decreasing amounts of glucose/phosphate. Also, variation of these two parameters among the H-ras cells was larger than the controls.

**Figure 4.1.12.1:** 2D plot of phosphate level vs. carbohydrate level. The phosphate level is calculated by integrated area of the phosphate band and the carbohydrate content by the intensity ratio 1045/1545.

**Figure 4.1.12.2:** 2D plot of RNA/DNA vs. glucose/phosphate for normal (f1) and H-ras transfected (f6) fibroblasts.
4.1.13 Cluster analysis

Cluster analysis was performed for different segments of the spectra to obtain the best results. Table 4.1.2 shows the results of our cluster analysis of FTIR spectra of controls (BM) and transformed cells (BMT). The regions correspond to symmetric and asymmetric (980-1350 cm\(^{-1}\)) stretching vibrations of phosphate were shown to classify both types of cells with 100% accuracy. The symmetric region (980-1149 cm\(^{-1}\)) alone provided 100% accurate classification of controls and transformed cells (shaded region). The region between 980-1759 cm\(^{-1}\) covering the phosphate, side chain and protein bands provided 100% classification for controls whereas only 87.5% was obtained for transformed cells. Other segments of the spectra provided 70-100% classification accuracy. Our results showed that the phosphate regions were the best for the classification of the two types of cells used in our study. The dendrogram showing the results of cluster analysis in the region between 980-1350 cm\(^{-1}\) is shown in Figure 4.1.13. The normal cells (controls) could be divided into two different subclusters having population of 42 and 58%. The transformed cells had a major and minor cluster with distribution similar to the controls. The heterogeneity was higher for normal cells than the transformed ones. The different growth stages might be responsible for the higher heterogeneity in the normal cells, which was reduced by retroviral infections.

Table 4.1.2: Summary of Cluster Analysis results performed for the rabbit bone marrow fibroblasts normal (BM) and transformed by MuSV (BMT) *

<table>
<thead>
<tr>
<th></th>
<th>980-1149</th>
<th>1151-1350</th>
<th>1340-1477</th>
<th>1475-1593</th>
<th>1591-1753</th>
<th>980-1350</th>
<th>980-1483</th>
<th>980-1593</th>
<th>980-1759</th>
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<tbody>
<tr>
<td>BM as BM</td>
<td>100%</td>
<td>85.5%</td>
<td>89%</td>
<td>96.4%</td>
<td>96.4%</td>
<td>100%</td>
<td>85.5%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>BM as BMT</td>
<td>0%</td>
<td>14.5%</td>
<td>11%</td>
<td>3.6%</td>
<td>3.6%</td>
<td>0%</td>
<td>14.5%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>BMT as BM</td>
<td>100%</td>
<td>100%</td>
<td>98.2%</td>
<td>69.6%</td>
<td>87.5%</td>
<td>100%</td>
<td>100%</td>
<td>89.3%</td>
<td>87.5%</td>
</tr>
<tr>
<td>BMT as BM</td>
<td>0%</td>
<td>0%</td>
<td>1.8%</td>
<td>30.4%</td>
<td>12.5%</td>
<td>0%</td>
<td>0%</td>
<td>10.7%</td>
<td>12.5%</td>
</tr>
</tbody>
</table>

* All wavenumbers are in cm\(^{-1}\)

BM as BM gives the correct percentage of identifying BM as BM. Similarly for BMT
Figure 4.1.13: Dendrogram presentation of controls (BM) and MuSV transformed (BMT) fibroblasts isolated from bone marrow of rabbits. These results were obtained by cluster analysis of 111 spectra in the region between 980-1350 cm$^{-1}$. For convenience, only the averages of seven clusters for controls and transformed cells are shown in the Figure.

4.1.14 Discussion

IR-spectra

With the aim of developing a unique technique for a laboratory diagnosis of malignant cells under controlled conditions, we analyzed the FTIR absorbance spectra of normal and malignant cells transformed by retroviruses and \textit{H-ras transfected cells in vitro}. Our results showed that, in general, the absorbance of the normal cells was significantly higher than that for malignant cells with some specific variations (Figures 4.1.2.2, 4.1.2.3 and 4.1.2.4).

The higher absorbance observed for normal cells compared to malignant fibroblasts indicate that there are changes in the overall cell metabolites between these two types.
In general, the absorbance of the peaks of the BM cells were significantly higher than those of the BMT cells using amide I normalization (Fig. 4.1.2.3). The transformation of fibroblast by MuSV124 is due to the *mos* oncogenic product, which is a Ser/Thr kinase [71]. NIH/3T3 fibroblast cells transformed by *ras* or *mos* oncogene had elevated glucose and the expression of glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GADPH) [120].

The differences in intensity between normal and malignant rabbit bone marrow cells were much higher than, murine fibroblasts transfected by MuSV and H-ras transfected mouse fibroblasts. This discrepancy is probably due to differences in the types of cells used, i.e., comparison between primary cells and cell lines in the murine fibroblasts transfected by MuSV and H-ras transfected mouse fibroblasts [87, 91].

**Phosphate level**

The informative PO$_2^-$ symmetrical and asymmetrical stretching vibrations, which occur between 980-1350 cm$^{-1}$, provide clues to qualitative and quantitative changes for phospholipids, nucleic acids and energy metabolites.

In our study, the absorption intensity of these vibrations for normal cells was larger than ras transfected types (illustrated by bands 13 and 17 in Figure 4.1.3). This feature was observed in all cases in the present work. The analysis of phosphate (sym. and asym.) bands has clearly shown that the total phosphate content is significantly higher in normal cells than the transfected types.

Spectral analysis showed that the levels of vital cellular metabolites in malignant cells transformed by retroviruses were lower than those in normal cells. The results are in agreement with previous findings indicating that ras-gene transfected fibroblasts showed a decrease in phosphate concentration relative to control cells [121, 91, 87]. In contrast, it has been reported that malignancies resulting from different causative factors other than retroviruses or oncogenes give rise to increases in phosphate levels [115, 122, 123]. It is possible that the differences in the results between our findings and previous studies reported by, Yang et al. [115] (human fibroblasts), Dukor et al. [122] (breast cancer) and Wong et al. [123] (human skin cells), could have their origin in the cause of the malignancy: in our study, the cells were transformed by a retrovirus (MuSV) or H-Ras, whereas
the cases reported by Yang et al., Dukor et al. and Wong et al., the cancers were of natural origin [115, 122, 123]. In addition, we used rabbit bone marrow cells, murine and mouse fibroblasts for our study, whereas the previously mentioned studies used human tissues.

**RNA/DNA ratio**

Our results indicate that the RNA/DNA ratio was increased in the malignant cells relative to normal fibroblasts. These results correlate well with reports available in the literature [116]. The increased RNA/DNA ratio in the H-ras transfected and retroviral-transfected cells might be explained by their increased metabolic and proliferative activity, relative to the controls. This differential cellular activity is expressed in their different doubling times, determined during the course of the FTIR measurements: 29.0 ± 0.8 h for the ras-transfected cells, and 34.25 ± 1.40 h for the controls as an examples. The ras mediated increase in the transcriptional activity can be the reason for the increase in the RNA/DNA for cancerous cells relative to the normals [124].

**Amide I/II ratio**

Higher amide I/II ratio for transformed cells (in the case of NIH/3T3-MuSV and BMT) can be understood by the effect of *mos* oncogeneic product resulting in increased DNA synthesis.

**IR-spectra (higher region)**

The absorption in the range 2800-3000 cm\(^{-1}\), which is due to strong absorption of CH\(_2\), CH\(_3\) stretching vibrations of phospholipids, cholesterol and creatine, is found to be lower for ras transformed and retroviral transformed cells than the normal fibroblasts. The reduction in the levels of phospholipids (CH\(_2\), CH\(_3\) stretching vibrations of phospholipids) in the ras transfected and retroviral cells accounts for our observation. The possible reason for higher absorbance over a wide wavelength range (with amide I normalization) for normal fibroblasts than the ras gene transfected may be due to the increase in the volume of the ras gene transfected fibroblasts, which has been studied in detail [125]. Lang et. al. [126] reported that the cell volume of H-ras transfected fibroblasts (2.70 ± 0.08 pl) was significantly larger than the cell volume of the controls (2.04 ±0.10 pl). The increase in cell volume
(without changing the sample thickness) effectively decreases the concentration (per unit volume) of the cellular contents, which can be correlated with the observed differences between the transfected and control fibroblasts. It is therefore interesting to note that there is a good agreement between the independent biochemical and the biophysical studies.

**Phospholipids**

Ras genes regulate key cellular signaling pathways [46]. Several lines of evidence indicated that among other cellular changes the phospholipid metabolism was altered as a result of oncogene induced transformation [127]. The phospholipid molecules and their metabolites are believed to participate in the processes of oncogene-induced transformation [128]. Momchilova [129] reported that all phospholipid fractions were reduced in ras-transfected fibroblasts except the phosphotidylethanolamine (PE). This trend was observed in our studies, also when vector normalization was applied to the spectra (instead of the amide I). This may explain our results that phosphate content was higher in normal cells than in the ras transfected cell lines.

**Glucose/phosphate and glucose/phospholipids**

The ratio of glucose/phosphate Figure 4.1.9.1 & Figure 4.1.9.2 and glucose/phospholipids Figure 4.1.10 was higher in controls relative to the transformed cells. But, the difference between them was not large, possibly the available glucose level remained the same in both types of cells. Since the energy status controls the production of other cellular metabolites, it can be concluded that the expression of H-ras has minimal effect on the cellular content of vital molecular constituents.

**Carbohydrate level**

The decrease in carbohydrate levels in ras transfected mouse fibroblasts may be the possible reason for the reduction in cell metabolites. This may account for the higher intensity observed for normal cells in comparison with the malignant samples in the entire region of the spectrum (using Amide I normalization) and the exact reasons are not yet clearly understood.
The linear correlation between carbohydrate and phosphate levels showed that the FTIR could reflect the situation in the cell, which could be derived from direct biochemical methods. The negative correlation existing between the RNA/DNA and glucose/phosphate can be a good indicator to identify the transforming status of the cells. It still remain to be seen if this can be applied to human tissues in the diagnosis of various types of malignancy where there is ambiguity in differentiating normal and cancerous cells.

**4.1.15 Summary and conclusions**

Our FTIR-MSP results on normal and MuSV infected fibroblasts isolated from bone marrow of rabbits, normal murine and H-Ras transfected fibroblasts cells and normal mouse and MuSV transformed fibroblasts has offered directions for the applications of advanced optical technology in the detection of malignancy caused by retrovirus and H-ras. It seems worthwhile to continue with the development of FTIR microspectroscopy for the purpose of malignant cell diagnosis and possibly even for the diagnosis of the cause of malignancy (at least in the case of H-ras and retroviruses).

Our study indicates that FTIR absorption in the ranges from 600-3200 cm\(^{-1}\) is lower for transformed fibroblast cells than the normal ones. The cellular contents of phospholipids such as PC, PS and PA may decrease in ras transfected cells, which correlate well with the reported literature. FTIR microscopy could differentiate H-ras transfected, retroviral transformed and their controls due to the intrinsic differences in the intensity. The various metabolites such as DNA, proteins, and lipids showed significant variation upon transformation. The decrease in the carbohydrate levels may be the possible reason for the overall decrease in the intensity of malignant fibroblasts relative to the controls. Hence, FTIR microscopy can be expected to become a powerful technique for the characterization of malignancy in vitro and in vivo in the future.
4.2) Colonic Tissues

The colon is the part of the digestive system where the waste material is stored. Cancer of the colon and rectum is the third leading cause of cancer in males and the fourth leading cause of cancer in females.

Benign tumors of the large intestine are called polyps. Malignant tumors of the large intestine are called cancers. Benign polyps can be easily removed during colonoscopy, and are not life threatening. If benign polyps are not removed from the large intestine, they can become malignant over time. Most of the cancers of the large intestine are believed to have developed from polyps. A malignant tumor consists of cancer cells, which have the ability to spread beyond the original site, and damage tissues and organs near the tumor.

Most professionals believe that colon cancer develops gradually over a period of years. Cells change from a precancerous state to a cancerous state during this time. Thus it is essential that patients undergo screening for early detection of precancerous conditions. When colon cancer is detected early, survival rates are much higher, so it is very important to develop a new diagnostic method for early detection of cancer.

The department of pathology Soroka Medical Center provided the samples we used in this study. Table 4.2.1. contains the list of samples obtained from patients, their sex, age, cancer and polyps stages. Several unique samples contain all three types of tissue, normal, polyp and cancer. Some samples contain only normal and cancer. The sign “+” means that the slide contains normal area, the sign “−” means that the slide does not contain area of the specified type of tissue. In this study we analyzed 24 patients and more than 500 spectra of normal, polyp and cancerous tissues have been measured. The serial number in the first column is referred to as the patient number in the figures.
Table 4.2.1: Sex, age and stages of polyp and cancer of all patients used in this study.

<table>
<thead>
<tr>
<th></th>
<th>Sex + Age</th>
<th>Normal</th>
<th>Polyp (dysplasia)</th>
<th>Cancer (differentiation)</th>
<th>Cancer (Astler-Coller)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M 79</td>
<td>+</td>
<td>Moderate villous</td>
<td>Early</td>
<td>B1</td>
</tr>
<tr>
<td>2</td>
<td>M 70</td>
<td>+</td>
<td>Moderate tubular</td>
<td>Villous</td>
<td>B2</td>
</tr>
<tr>
<td>3</td>
<td>M 44</td>
<td>+</td>
<td>-</td>
<td>Well to Moderate</td>
<td>B2</td>
</tr>
<tr>
<td>4</td>
<td>M 89</td>
<td>+</td>
<td>-</td>
<td>Moderate</td>
<td>B2</td>
</tr>
<tr>
<td>5</td>
<td>M 68</td>
<td>+</td>
<td>-</td>
<td>Well to Mod.</td>
<td>B2</td>
</tr>
<tr>
<td>6</td>
<td>M 44</td>
<td>+</td>
<td>-</td>
<td>Moderate</td>
<td>B2</td>
</tr>
<tr>
<td>7</td>
<td>F 74</td>
<td>+</td>
<td>-</td>
<td>Moderate</td>
<td>B2</td>
</tr>
<tr>
<td>8</td>
<td>F 84</td>
<td>+</td>
<td>Moderate tubular</td>
<td>Advanced</td>
<td>C2</td>
</tr>
<tr>
<td>9</td>
<td>F 47</td>
<td>+</td>
<td>-</td>
<td>Moderate</td>
<td>C2</td>
</tr>
<tr>
<td>10</td>
<td>F 87</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>C2</td>
</tr>
<tr>
<td>11</td>
<td>M86</td>
<td>-</td>
<td>Mild-Moderate</td>
<td>Tubular</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>M66</td>
<td>-</td>
<td>Moderate</td>
<td>Tubular</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>F69</td>
<td>-</td>
<td>Moderate-Severe</td>
<td>Tubular</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>F62</td>
<td>-</td>
<td>Mild</td>
<td>Moderate</td>
<td>C2</td>
</tr>
<tr>
<td>15</td>
<td>M78</td>
<td>-</td>
<td>Mild</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td>F76</td>
<td>+</td>
<td>Moderate</td>
<td>Tubular</td>
<td>Moderately to poorly</td>
</tr>
<tr>
<td>17</td>
<td>F76</td>
<td>+</td>
<td>Moderate</td>
<td>Tubular</td>
<td>Moderately to poorly</td>
</tr>
<tr>
<td>18</td>
<td>F76</td>
<td>+</td>
<td>Moderate</td>
<td>Tubular</td>
<td>Moderately to poorly</td>
</tr>
<tr>
<td>19</td>
<td>F41</td>
<td>+</td>
<td>Moderate</td>
<td>Tubular-Villous</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>M66</td>
<td>+</td>
<td>Mild-Moderate</td>
<td>Tubular</td>
<td>-</td>
</tr>
<tr>
<td>21</td>
<td>F68</td>
<td>+</td>
<td>Moderate</td>
<td>Tubular, villous</td>
<td>-</td>
</tr>
<tr>
<td>Sex + Age</td>
<td>Normal</td>
<td>Polyp (dysplasia)</td>
<td>Cancer (differentiation)</td>
<td>Cancer (Astler-Coller)</td>
<td></td>
</tr>
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<td>--------------------------</td>
<td>-----------------------</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>-</td>
<td>Moderate</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>-</td>
<td>Moderate</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>-</td>
<td>Mild to Moderate</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

### 4.2.1 Histological images of Normal, Polyp and Malignant human colonic tissues

Figure 4.2.1a presents histological cross section image of formalin-fixed human normal colonic tissues. The thin microvascular vessels pass vertically between crypts in the normal lamina propria. The normal colonic mucosa (Figure 4.2.1b) is made up of epithelial lined crypts that are surrounded by lamina propria. The histological section is oriented in such a way that simple columnar surface epithelium facing the lumen is at the left of the Figure and the lamina propria in which they are situated at the right of the Figure. The mucosal crypts are parallel to one another and unbranched. The adenomatous epithelium preserves mucus production-goblet cells. In this field there is a marked decrease in mucus production. There is focal loss of nuclear polarity, and an increase in nucleocyttoplamic ratio. The polyp lesions (Figure 4.2.1c) shows different morphology compared to normal, appearing as pedunculated growths above the surrounding normal tissue surface. The Figure displays adenomatous polyp with tubular adenoma. The neoplastic glands are forming tubules. Figure 4.2.1d gives a low power view of adenocarcinoma, a malignant epithelial tumor. The gland shows cribriform pattern, with loss of mucin production and foci of necrotic debris. The adenocarcinoma glands (grade II) are irregular and torous.

### 4.2.2 Morphological Changes for Normal, Polyp and Malignant Lesions in Human Colonic Tissue

Histological cross section of formalin-fixed human colonic tissue is shown in Figure 4.2.2. The encircled areas in Figure 4.2.2a-c show the site covered by FTIR-MSP measurements in this study. Figure 4.2.2a shows the crypts of the normal colonic mucosa. The epithelium surrounds a central opening and the lumen of the crypt.
Figure 4.2.1: Histological image of formalin fixed normal human colonic tissue staine with hematoxylin-teosin (a) cross section image (b) lateral section image (c) Polyp cross section (d) cross section of colonic cancerous tissue.
Figure 4.2.2: Histological image cross sections of formalin fixed human colonic tissue stained with hematoxylin-teosin (a) normal (b) Polyp (c) malignant. Encircled areas show the selected sites for FTIR-MS measurements.
The normal epithelial cells show a single row of regular cells with small nuclei in the periphery of the cell and abundant cytoplasm. The nuclear to cytoplasm ratio is low. The extra cellular matrix (lamina propria) around the epithelium consists of scattered lymphocytes and granulocytes (inflammatory cells)(dark dots). Figure 4.2.2b shows the neoplastic epithelial lesion (tubular adenoma) showing a mild change from the normal architecture with a slightly irregular crypt, and has crowded nuclei. The nuclear to cytoplasm ratio is increased compared with normal colon tissue, while the lumen is narrowing. Figure 4.2.2c shows the adenocarcinoma of the colon showing stratification of hyperchromatic nuclei with marked atypical cells. The glands become irregular and situated back to back. The lumen size is uneven containing cellular debris. There is desmoplastic stromal reaction with a secondary inflammatory reaction.

4.2.3 FTIR Microspectroscopy (FTIR-MSP) of Normal polyp and Malignant Human Colonic Tissues in the Lower Wavenumber Region.

The microscopic mid-IR spectra of normal, adenomatous polyp and malignant cells from biopsy tissue samples of three different patients of varying disease stages are shown in the Figure 4.2.3 in the spectral range 900-1800 cm\(^{-1}\). The patients a (patient No 1), b (patient No 2), and c (patient No 8) (From table 4.2.1) are classified as early, moderate and advanced stages of malignancy respectively as assigned by our group pathologists. Due to the limited availability of all three types of cells (normal, polyp and malignant) on any given tissue sample, only three cases are given in this work. The spectra were normalized to amide I. The absorption due to normal tissue was higher than polyp and cancerous types in the entire region of the spectrum in all three patients accompanied with some specific changes. In the early case (patient a), the absorbance of adenomatous polyp was lower than the malignant cells. This trend was reversed in the advanced case (patient c). No significant frequency shifts were observed between normal and cancerous tissues in the entire region (600-4000 cm\(^{-1}\)). The selection of amide I or vector normalization was discussed in section 2.2.11.4.

The intensity differences for normal, and cancerous for amide II band was not significant in all cases. The weaker amino acid side chain vibrations from peptides
and proteins at 1456 and 1401 cm\(^{-1}\) are associated with the asymmetric and symmetric CH\(_3\) bending vibrations [34].

There was a distinct change in the pattern in the region between 1000-1200 cm\(^{-1}\) for normal and abnormal tissue samples, which include polyp and malignant tissues. In the spectrum of normal cells, splitting can be clearly observed in the region 1000-1150 cm\(^{-1}\), where it disappeared in the case of adenomatous polyp and malignant cases. This has been observed in most of the cases studied by our group.

### 4.2.4 Spectral Analysis and Band Fitting

Typical spectral analysis and band fitting performed for the region 600-1730 cm\(^{-1}\) in the present work is shown in Figure 4.2.4.1 (shown for patient number 8). The same fitting procedure has been used for the normal, polyp and the malignant sections. The parameters used in the fitting procedure were discussed in section 3.5.1.

Figure 4.2.4.2 displays the analytical areas for the different peaks between 950-1600 cm\(^{-1}\) deduced from the fitting analysis in the case of patient number 8. Out of 16 peaks shown in Figure 4.2.4.2, the bands for phosphate (peaks 13 & 17, due to phosphate group symmetric and asymmetric stretching vibrations respectively) show remarkable differences between normal and cancerous types.

### 4.2.5 Phosphate Levels

The analytical areas under the phosphate absorption were calculated for normal and malignant tissues for our patients and they are presented in Figure 4.2.5. The Figure gives a clear picture of the variation in the phosphate content for normal, polyp and malignant tissues from all patients. In all patients, the phosphate content of normal tissue was higher than the malignant tissues. The phosphate levels were found to be lower in cancerous tissues in younger compared to elderly patients. The analysis of phosphate bands arising from symmetric and asymmetric stretching vibration bands, has clearly shown that the total phosphate content is significantly higher in normal tissues than in the three cancer types, early moderate and advanced cancers. The error bar was calculated for each sample (normal or malignant) separately for all the patients. It is also clear from Figure 4.2.5 that the diversity among polyp was larger than the malignant and the controls.
Figure 4.2.3: FTIR microspectroscopy of normal, polyp and malignant tissue samples from three patients in the range 900-1800 cm$^{-1}$. The labels a, b and c represent early, moderate and advanced stages of malignancy respectively. All the spectra are normalized to amide I consistently.
Figure 4.2.4.1: Typical band fitting analysis in the wavenumber region 600-1730 cm$^{-1}$ for patient number 8 (see table 4.2.1). The dots represent the data points and the blue solid line represents the overall fit.

Figure 4.2.4.2: Analytical areas of the absorption bands observed in the region 950-1580 cm$^{-1}$ for patient number 8. The analytical areas were calculated for 5 normals and 5 cancerous spectra taken from different sites of the same sample.
**Figure 4.2.5:** Phosphate as a biological marker derived from the FTIR spectra for 24 patients. The phosphate content is calculated as the sum of symmetric and asymmetric bands of phosphate group. PeakFit software was used for this purpose.

### 4.2.6 RNA/DNA Ratios

Our analysis presented in Figure 4.2.6 indicates that the RNA/DNA ratio was lower for normal cells in comparison to polyp and malignant types. Also, it is interesting to note that the RNA/DNA ratio was nearly constant for normal and polyp samples. But, there was marked variation among the malignant samples obtained from different patients.

Except for the cases of patient numbers 1 and 4, the RNA/DNA ratio was higher for malignant tissues than the normal samples. Our analysis of RNA/DNA for the patients showed that in the moderate and advanced cases, the RNA/DNA was higher for malignant tissues than the normal type, which may be a good parameter for diagnostic purposes.
Figure 4.2.6: The absorbance ratio at I(1121)/I(1020) is presented as RNA/DNA for all the 24 patients.

4.2.7 FTIR Microspectroscopy (FTIR-MSP) of Normal and Malignant human colonic tissues in the Higher Wavenumber Region.

Figure 4.2.7 shows the higher wavenumber region between 2600-3800 cm$^{-1}$ for normal, polyp and cancerous tissues for three patients a (early), b (moderate) and c (advanced). Here also, the absorbance magnitude of normal tissue was higher than the polyp and cancerous types.

4.2.8 Cholesterol, Phospholipids and Creatine

The integrated areas (for peaks I and II in Figure 4.2.7) at 2848 cm$^{-1}$ and 2916 cm$^{-1}$ for the first ten patients are presented in Figure 4.2.8 a-b. The integrated absorbance for normal tissue was higher than cancer types in most of the cases, regardless of the stage of cancer according to the three cases (1 (early), 2 (moderate) and 8 (advanced) as indicated in Table 4.2.1). The difference between normal and malignant tissue types is larger in early case compare to moderate and advanced cases.
Figure 4.2.7: Infrared microspectroscopy in the region 2600-3800 cm\(^{-1}\) of normal, polyp and malignant tissues of human intestine. The patient labels a, b and c indicates early, moderate and advanced stages of the malignancy.
Figure 4.2.8: (a) Integrated absorbance of peak I (2848 cm$^{-1}$) for the first ten patients (table 4.2.1). The blue and red symbols are normal and cancerous respectively. (b) Integrated absorbance of peak II (2916 cm$^{-1}$) for the first ten patients (table 4.2.1).
4.2.9 Carbohydrate Levels

Our results indicated that carbohydrate (including glycogen) levels shown in Figure 4.2.9 were reduced in cancerous tissues in comparison to normals, regardless of the stage of malignancy, (patient No. 1 (early), 2 (moderate) and 8 (advanced)). The difference in carbohydrate level between normal and cancer is highest in patient No. 8 (c) who is in the advanced stage compared to patients 1 (early) and patient 2 (moderate). It may be that the carbohydrate absorption or metabolism is affected in cancerous tissue in the advanced stages.

The levels of carbohydrate for normal samples for both age groups (young and elder) were in the same range. However, carbohydrate levels did not change considerably between males and females.

4.2.10 Glucose/Phosphate Ratio

The ratio of glucose/phosphate shown in Figure 4.2.10 shows that the glucose/phosphate ratio was higher for normal than malignant tissues, similarly in the case of phosphate levels. Patient 1 (early cancer) is in disagreement with the other nine patients and it maybe due to grading (early) for the cancer as assigned by the pathologist.

4.2.11 2D Plot of Carbohydrate vs. Phosphate contents

It is interesting to find the correlation between different metabolites among the various cell types. The two-dimensional plot shown in Figure 4.2.11 gives the relation between carbohydrate and phosphate content in normal, polyp and malignant cells. The change in carbohydrate levels (calculated as the intensity ratio at (1045/1545) X100) is expected to have impact on phosphate levels as the energy metabolism governs the synthesis of various metabolites containing phosphate group. It is clear from the Figure that there is a linear correlation between these two metabolites. The normal samples had highest carbohydrate and phosphate contents. The distinction between polyp and malignant samples was absent. The perfect mixing of these two types categorize them to a single family in this figure.
Figure 4.2.9: The intensity ratio at (1045/1545) X 100 for the first ten patients (table 4.2.1).

Figure 4.2.10: The intensity ratio at (1030/1080) for the first ten patients (table 4.2.1).
Figure 4.2.11: 2D plot of carbohydrate vs. phosphate content for 24 patients. The carbohydrate content is measured as the ratio of the absorbance at (1045/1545)X100.

4.2.12 Classification of Normal and malignant samples by LDA

In order to differentiate between normal and cancerous tissues we used the LDA method. The preliminary results of LDA are highly encouraging in discriminating normal from malignant cells. A summary of the different feature sets is shown in Table 4.2.2. The probabilities of classification using seven sets of features for normal and cancer tissues are shown in Table 4.2.3. The best results were obtained for set 1 (phosphate bands labeled 13 and 17, Table 4.2.2) with a success rate of 86.2% and 91.6% for normal and cancer tissue, respectively. Although the success rate of the LDA based classifier is high, a careful analysis of Table 4.2.3 reveals that about 8.4% of the cancer cases might be classified as normal while up to 13.8% of the normal cases might be classified as cancer.
Table 4.2.2: Feature combinations of the Linear Discriminant Analysis.

<table>
<thead>
<tr>
<th>Feature Description</th>
<th>Vector size</th>
<th>Feature Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate bands labeled 13 and 17</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Integrated area of peaks I (2848 cm$^{-1}$) and II (2916 cm$^{-1}$)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>The intensity ratio at I (1121) / I (1020) and I (1045) / I (1545)</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>The intensity ratio at I (1045) / I (1545) and summed analytic areas of the phosphate bands labeled 13 and 17</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Phosphate bands labeled 13 and 17 and the intensity ratio at I (1045) / I (1545)</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Phosphate bands labeled 13 and 17 and RNA</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Phosphate bands labeled 13 and 17 and the intensity ratio at I (1121) / I (1020)</td>
<td>3</td>
<td>7</td>
</tr>
</tbody>
</table>

Table 4.2.3: FTIR assessment for Cancer: the percentage of correct and incorrect test diagnoses, using Linear Discriminant Analysis (LDA).

<table>
<thead>
<tr>
<th>Feature Identification</th>
<th>Normal as Normal</th>
<th>Cancer as Cancer</th>
<th>Normal as Cancer</th>
<th>Cancer as Normal</th>
<th>Total*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>86.2</td>
<td>91.6</td>
<td>13.8</td>
<td>8.4</td>
<td>88.9</td>
</tr>
<tr>
<td>2</td>
<td>93.2</td>
<td>55.4</td>
<td>6.8</td>
<td>44.6</td>
<td>74.3</td>
</tr>
<tr>
<td>3</td>
<td>84.4</td>
<td>84.4</td>
<td>15.6</td>
<td>15.6</td>
<td>84.4</td>
</tr>
<tr>
<td>4</td>
<td>84.6</td>
<td>89.6</td>
<td>15.4</td>
<td>10.4</td>
<td>87.1</td>
</tr>
<tr>
<td>5</td>
<td>80.4</td>
<td>78.2</td>
<td>19.6</td>
<td>21.8</td>
<td>79.3</td>
</tr>
<tr>
<td>6</td>
<td>77.0</td>
<td>90.8</td>
<td>23.0</td>
<td>9.2</td>
<td>83.9</td>
</tr>
<tr>
<td>7</td>
<td>83.2</td>
<td>90.2</td>
<td>16.8</td>
<td>9.8</td>
<td>86.7</td>
</tr>
</tbody>
</table>

- Total is the average of positive identification for both normal and cancer types (columns 2 & 3).
4.2.13) ANN analysis of the results

The basic principles of the ANN analysis have been described earlier in section 3.5.4. In this study, the FWT, proposed by Mallat and Zhong [113] is applied. The sampled spectrum is decomposed into an orthogonal set of waveforms that are the dilations, translations and modulations of the Coiflet wavelet (mother wavelet). The Coiflet wavelet was chosen because in practice it showed better results than other common wavelets that were tested. The wavelet transform is computed by convolving the spectrum with these dilated wavelets. The wavelets coefficients of the different scales offer a compact representation of the spectrum signal, as shown in Figure 4.2.13. It is evident that the transform involves differentiation and progressive smoothing. Details of the peaks are gradually lost as the downward slopes of the wave are being picked up at higher scales. The number of scales should be chosen by searching for the optimal signal representation. It was found that scales higher than the first five do not add significant information about the spectrum (Fig. 4.2.13). In the present case, the performance of the MLP based classifier for different sets of wavelet coefficient features was examined (Table 4.2.4).

The wavelet coefficients were located around the two main peaks (amide I and II) of the spectra. The data employed in this work was extracted from twenty-four patients. The data sets consist of three groups: the cancer group (83 records extracted from 11 patients), the control group (109 records extracted from 16 patients) and the polyp group (106 records extracted from 12 patients). A total of 298 records were available. The obtained samples were classified with the help of an expert pathologist and confirmed by clinical diagnosis using standard pathological methods. In ANN analysis, training and test sets were selected randomly from the same data sets. Seventy percent of each set was employed for training and the remainder for test. In addition, the simulations were repeated 100 times, with the same networks parameters but with different sets of randomly selected training vectors, and the results were averaged.

4.2.14 Classification of Normal, Polyp and malignant samples by ANN

A summary of the results obtained with the MLP based classifier for the five sets of features (Table 4.2.4) is shown in Table 4.2.5. The feature combinations were
selected based on the biomarkers in their respective spectral regions. For example, the spectral region between 1055-1719 cm$^{-1}$ include the phosphate (both symmetric and asymmetric stretching bands) and total protein content of the cells.

**Figure 4.2.13**: Example of wavelet transform of FTIR spectra. The original signal is shown at the bottom of the panel and above it are the successive scales of the wavelet coefficients.

The best results were obtained for the MLP with a set composed of thirteen input features coefficients located between 2465 to 2963 cm$^{-1}$ which correspond to the absorption of phospholipids (set 1 Table 4.2.5).
Table 4.2.4: Feature combinations used in ANN analysis

<table>
<thead>
<tr>
<th>Feature Description</th>
<th>Vector size</th>
<th>Feature Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coefficients from 2465 to 2963 cm⁻¹</td>
<td>13</td>
<td>1</td>
</tr>
<tr>
<td>Coefficients from 1055 to 1719 cm⁻¹</td>
<td>17</td>
<td>2</td>
</tr>
<tr>
<td>Five coefficients from 2631 to 2797 cm⁻¹ and nine from 1221 to 1553 cm⁻¹</td>
<td>14</td>
<td>3</td>
</tr>
<tr>
<td>Coefficients from 1055 to 2963 cm⁻¹</td>
<td>47</td>
<td>4</td>
</tr>
<tr>
<td>Thirteen coefficients from 2465 to 2963 cm⁻¹ and seventeen from 1055 to 1719 cm⁻¹</td>
<td>30</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 4.2.5: FTIR assessment for Normal, Cancer and Polyp diagnosis: the percentage success of the test results. The best results are shadowed.

<table>
<thead>
<tr>
<th>Feature Identification</th>
<th>Normal</th>
<th>Cancer</th>
<th>Polyp</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>89.4</td>
<td>83.1</td>
<td>81.3</td>
<td>84.8</td>
</tr>
<tr>
<td>2</td>
<td>88.4</td>
<td>84.4</td>
<td>80.1</td>
<td>84.3</td>
</tr>
<tr>
<td>3</td>
<td>90.7</td>
<td>83.9</td>
<td>79.5</td>
<td>84.8</td>
</tr>
<tr>
<td>4</td>
<td>89.0</td>
<td>83.6</td>
<td>79.5</td>
<td>84.1</td>
</tr>
<tr>
<td>5</td>
<td>87.6</td>
<td>83.6</td>
<td>80.3</td>
<td>83.9</td>
</tr>
</tbody>
</table>

The MLP-based classifier resulted in sensitivity values such as 89.4%, 83.1% and 81.3% for normal, cancer and polyp tissues, respectively. Although the sensitivity values obtained using this classifier was high, a careful analysis of Table 4.2.5 revealed that false positive for normal was up to 10.6%, which might be classified as either cancer (2.0%) or polyp (8.6%) as shown in table 4.2.6. On the other hand the sum of false negative and false positive for polyp group was about 18.6% (about 9.7% of the polyp cases might be classified as normal while up to 8.9% might be classified as cancer). It is important to note, that when compared with the results obtained by LDA [26, 27] method for classification of normal and malignant samples from colon cancer patients, these results have a good agreement and showed greater consistency.
Table 4.2.6: FTIR assessment as a confusion matrix for feature identification No. 1 in table 4.2.4, the percentage of correct and incorrect test diagnosis for Normal, Polyp and Cancerous colonic tissues.

<table>
<thead>
<tr>
<th>Estimated Source</th>
<th>Normal</th>
<th>Cancer</th>
<th>Polyp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>89.4</td>
<td>2.0</td>
<td>8.6</td>
</tr>
<tr>
<td>Cancer</td>
<td>2.5</td>
<td>83.1</td>
<td>14.4</td>
</tr>
<tr>
<td>Polyp</td>
<td>9.7</td>
<td>8.9</td>
<td>81.3</td>
</tr>
</tbody>
</table>

4.2.15 Discussion

FTIR microspectroscopy

The FTIR-MSP spectra of normal, polyp and malignant cells from tissue samples of different colon cancer patients showed the presence of two families. These are normal and abnormal which include polyp and malignant cells. The splitting pattern of multiple overlapping peaks observed in the range between 1000-1150 cm\(^{-1}\) for normal cells and its absence in polyp and malignant cells clearly indicates that FTIR-MSP is capable of detecting the early malignancy among colon cancer patients using biopsied tissue samples. This observation has been consistent in many of our samples, which have been studied and provide a reliable difference in the spectral pattern for diagnostic purpose. Our analysis showed that the spectral differences between polyp and malignant were not significant. Hence, more sophisticated computational tools are necessary to get a good differentiation between these two kinds of cells. The higher absorbance of normal relative to polyp and malignant cells may be attributed to the difference in the cellular lifecycle in the tubular gland (called crypt) between normal and malignant cells.

Biological markers:

Phosphate levels

Use of biological markers derived from IR spectroscopy in the identification of normal and malignant cells has been extensively investigated by many groups [26, 91, 115, 122, 123]. The phosphate levels reveal the metabolic turn over, as it consists of energy producers such as ATP & GTP and other biomolecular components, which include phospholipids, nucleic acids (DNA and RNA) and phosphorylated proteins.
The informative $\text{PO}_2^-$ symmetrical and asymmetrical stretching vibrations, which occur between 980-1350 cm$^{-1}$, provide clues to qualitative and quantitative changes for phosphate containing compounds (phospholipids and nucleic acids). In our study, the absorbance intensity of these band structures for normal tissue was higher than cancerous types (as illustrated by bands 13 and 17 in Figure 4.2.5) in all patients. Mantsch and co-workers [130] cautioned that in some studies the spectral differences observed between normal and malignant tissues could be due to collagen content. The examination of the measured areas of the tissue sample in all the cases confirmed that the interference due to collagen was very minimal and hence our spectral changes are not due to collagen content. Also to investigate the interference due to mucins layer present in the colonic tissue, the measurements were taken at different aperture values. Our results showed that the intensity changes at different values were not significant and the contribution due to mucins layer to the observed spectra was negligible. In a previous study reported by Rigas [131] the malignant tissues displayed decreased intensity of the $\nu_{\text{as}}\text{PO}_2^-$ band and increased intensity of the $\nu_{\text{sy}}\text{PO}_2^-$ band, when compared to normal (for colon cancer) tissues. They also reported that the intensity of these peaks varied from patient to patient and also within the sample. In our study, such discrepancies were not found, possibly the regions of colon and rectal may behave differently. Also, when the analytical areas for these two bands were summed up, the same trend was repeated (Figure 4.2.5). The analysis of phosphate (symmetric and asymmetric) bands has clearly shown that the total phosphate content is significantly higher in normal tissues than polyp and malignant types. Also, the difference in total phosphate level between normal and malignant was higher than the differences reported by Rigas [123], and this enhancement may arise from the fact that phosphate level is the summation of larger number of biomolecules having phosphate group. Phosphate is one of the abundant metabolites, which clearly differentiates normal samples from both polyp and malignant classes. But, it fails to give a separation between polyp and malignant cells from the tissue samples of colon cancer patients. The lower phosphate content for patient (sample Nos 11 and 14) Figure 4.2.5 is possibly due to reduction in total carbohydrate level, which is clear from the 2D plot shown in Figure 4.2.11.

Other cancer forms e.g. sarcoma [115], breast cancer [122] and skin cancer [123] show an increase in the phosphate concentration relative to the normal tissues.
Colonic adenocarcinoma shows an entirely different behavior. Our results can be explained by having a better understanding of histogenesis of colonic adenomas. Generally, in normal crypt, the epithelial cells proliferate in the bottom portion and move further with differentiation and finally exit ending up in apoptosis. The recent model (schematically shown in Figure (4.3.1) on histogenesis of colonic adenomas given by Moss et al [132] claims that in adenomas, the proliferation of cells is predominant at lumen and apoptosis at the base, a complete reversal of normal pattern. It is interesting to speculate that the drastic decrease in cellular contents for cancerous tissues (as shown by our results) can be correlated to the apoptotic stage of cancerous tissues. This explains why our results are opposite to the findings for other tissue types. Additional evidences are necessary to substantiate our results by having more microscopic FTIR data on samples from different sites of the adenomatous crypt in patients. This issue will be addressed in more details in section 5.3, were we measured bottom and top areas from normal and cancerous crypts.

**RNA/DNA ratio**

RNA/DNA (Figure 4.2.6) can distinguish only normal from abnormal types such as polyp and malignant. Again, the mixing of both polyp and malignant does not provide unambiguous identification of these two classes. In conclusion, presently the biological markers were not useful in correct classification of polyp and malignant types. Our results showed that in eight out of ten patients, the RNA/DNA ratio (Figure 4.2.6) increased from normal to cancerous stage. These results correlate well with reports available in the literature [116].

**FTIR spectra of the higher wavenumber region**

The region between 2800-3500 cm⁻¹ is due to strong absorption of CH₂, CH₃ stretching vibrations of phospholipids, cholesterol and creatine. Creatine and cyclocreatine have been shown to inhibit the growth of a variety of human and murine tumors [133]. Antiproliferative effect of creatine is shown to be effective in mice carrying a human colon adenocarcinoma (LS 174T) [133]. As in the case of phosphate bands, the intensity of higher wavenumber region for normal tissue was higher than cancer. Our results indicate that the creatine levels may be lower in cancer types compared to normal ones in accordance with the above studies.
Our results indicated that carbohydrate (including glycogen) levels were reduced in cancerous tissues in comparison to normals. This is in agreement with the decrease in phosphate levels presented in Figure 4.2.5. It is surprising that the carbohydrate (glycogen) levels decrease in normal tissue relative to the cancerous type in the case of rectal region of the intestine [134], for which the reasons are not clearly understood and possibly it might arise from the different biological samples in the two cases.

The influence of the parameters age, stage of malignancy and sex on the biological metabolites

The vital parameters such as age, stage of malignancy and sex are not studied in detail in terms of biochemical changes occur in colon cancer. Hence, we decided to analyze the available FTIR data for the first ten patients (in table 4.2.1) with respect to age and sex. In the group of ten patients studied in detail, there were three young (40-45; patients 3, 6, 9) and seven elderly (70-90) patients. There were six male and four female patients. The various biochemical markers were analyzed to get correlation between the different age and sex groups.

Patient number 9 is young in the advanced stage of malignancy having lowest phosphate level in the normal tissue, highest RNA/DNA and lowest carbohydrate content in the malignant tissue. In patient number 1 (exactly opposite to patient No 9) who is elderly in the early stage of malignancy was found to have highest phosphate level in the normal tissue, lowest RNA/DNA and highest carbohydrate content in the malignant tissue. The comparison between patient number 1 (old and early stage of malignancy) with patient number 6 (young and moderate stage of malignancy) also followed the same trend. This data shows that there is a good correlation between age & stage of malignancy and the composition of different biomolecular components in the tissue of colon cancer patients. Similarly, patient number 6 who is young having moderate stage of colon cancer was found to display the largest difference in carbohydrate and phosphate levels between normal and malignant tissues, whereas patient number 4, old being in the moderate stage showed the reversal of this trend. This also confirms that age has significant effect on different biochemical markers studied in the case of colon cancer patients using FTIR microscopy. To substantiate this point, it is essential to do a detailed study comprising larger number of patients.
Linear Discriminant Analysis (LDA)

In order to differentiate between normal and cancerous tissues we used the LDA method. The best results were obtained for set 1 (phosphate bands labeled 13 and 17, Table 4.2.2) with a success rate of 86.2% (specificity 0.862) and 91.6% (sensitivity) for normal and cancer tissue, respectively. Although the success rate of the LDA based classifier is high, a careful analysis of Table 4.2.2 reveals that about 8.4% of the cancer cases might be classified as normal while up to 13.8% of the normal cases might be classified as cancer.

Artificial Neural Network (ANN)

As the FTIR spectral differences and the biological markers were not sufficient to get good classification between polyp and malignant samples, we investigated the use of ANN, which is an advanced computational method. The results of these calculation show that the correct diagnosis for normal cells was higher than polyp and malignant types (Table 4.2.4). Therefore, normal can be differentiated from abnormal with good accuracy (98.4%). Polyp and malignant classes have similar classification percentage (83.1% and 81.3% respectively) with various features. But, using selected regions of the spectrum provided better results in the case of polyp in comparison to malignant type. The false negative diagnosis of polyp was about 20% indicating that progress is still to be made to enhance the accuracy of identifying polyp with high percentage. ANN gives better classification for polyp and malignant types than the other computational methods such as Cluster Analysis and LDA.

The sensitivity of standard pathological methods using biopsy in positive identification of adenoma is 83.6 % and our method is comparable to the “Gold Standard” approach [135]. In summary, our results with limited database are encouraging. It is expected to improve dramatically with large database and suitable fine tuning changes in the computational methods.

4.2.16 Conclusions

The results obtained on limited data reported in this study support the idea that major biochemical changes are taking place in the cells undergoing transformation from normal to cancerous state. Cancerous colonic epithelial cells
show a systematic decrease in total carbohydrate, phosphate and possibly creatine contents. It seems that the phosphate stretching modes could be useful as infrared spectroscopic markers to discriminate between spectra of normal and cancerous colonic tissues. Also, it is important to identify the special patterns of vibrational modes characteristic to various states of malignancy. With even limited number of patients it could be shown that age and stage of malignancy have dramatic effect on FTIR markers reported in this study. Such study is essential for evaluating the potential of IR microscopy as a new tool for early detection of cancer and to get a better insight on the differences between normal, polyp and cancerous cells. Our initial results on mathematical analysis of FTIR spectra on normal, polyp and malignant tissue samples showed good classification giving room for detailed investigation in the future.

Our study with of normal, polyp and malignant colonic tissues has clearly shown that FTIR microscopy can be developed for the purpose of diagnosis. However large database is essential to turn this methodology into novel diagnostic technique for colon cancer using FTIR. Studies with larger number of human samples and use of state of the art imaging techniques may improve the results in the early diagnosis of colon cancer in the future.

4.3 Crypt

Human small intestinal surface consists of small crypts [78]. The intestinal crypts contain mucus secreting goblet cells in large number [78]. The epithelial cells in the normal crypt continuously undergo mitosis at the bottom of the crypt and move gradually along the basement membrane upwardly and finally disposed into the intestinal secretions (Figure 4.3.1) [136, 137, 138]. It is speculated that the direction of the migration of cells reversed in the adenomatous crypt [139].

To date, there is no spectroscopic investigation on cellular activity in the human crypt in the intestine. In this section, we provide microscopic FTIR proof for the variation in the cellular activity at different sites in the human crypt foci in the intestine. Our results suggest that remarkable differences in the spectra are observed for the cells at the bottom and top of the crypt.
4.3.1 FTIR Microspectroscopy (FTIR-MSP) of Normal Crypt.

Figure 4.3.2 shows the FTIR-MSP spectra of normal crypts from two different patients. The absorbance for the cells at the bottom (blue lines) site was higher than the top (green lines). The absorbance differences between two different crypts for both bottom and top sites were small and hence there was good agreement between various patients. Notable pattern changes were also observed between the bottom and top sites. The cells at bottom showed a doublet structure pattern between 1000-1100 cm\(^{-1}\) at 1050 cm\(^{-1}\) and 1078 cm\(^{-1}\), which was absent for the cells at the upper portion of the crypt. This was observed in all five patients. There was a frequency shift of 4 -5 cm\(^{-1}\) in the amide I peak between the two sites for the young (47 years) patient whose grading of malignancy was declared as advanced by the pathologist. This can be clearly seen in second derivative spectrum shown in the Figure 4.3.3. These frequency shifts were observed in another old and having advanced stage of malignancy. The frequency shifts were observed also for amide II, but they were not significant.

FTIR-MSP spectra in the range between 2600-3600 cm\(^{-1}\) showed no significant changes in the absorbance between the two sites. The region beyond 3000 cm\(^{-1}\) is not
considered for analysis, as the absorption due to water is higher and could not be attributed to the samples. To ensure that our results are independent of methods of treating the spectra, we employed also vector normalization to all the spectra. The vector normalized spectra are presented in Figure 4.3.4. As it can be seen, the absorbance for cells at the bottom was again higher than the top portion of the normal crypt. These spectral pattern changes observed using amide I normalization were maintained and in addition, the disappearance of a hump near 1000 cm$^{-1}$ for the cells at the top of the crypt was clear with vector normalization.

Figure 4.3.2: FTIR microspectroscopy of epithelial cells at the bottom (a, b) and top (c, d) of a normal crypt in the region 800-1800 cm$^{-1}$. This sample was obtained from a young patient having advanced stage of the malignancy. Amide I normalization was applied to the spectra shown in this Figure.
Figure 4.3.3: Second derivative spectra for the sample shown in the Figure 4.3.1.

Figure 4.3.4: FTIR-MSP spectra shown in Figure 4.3.1 but with vector normalization.
Figure 4.3.5: FTIR-MSP spectra of epithelial cells at the bottom of normal (a, b) and malignant (c, d) crypts from a patient in the region 800-1800 cm$^{-1}$.

4.3.2 FTIR Microspectroscopy (FTIR-MSP) of Normal and cancerous Crypt.

Figure 4.3.5 shows the FTIR-MSP of the bottom portion of normal and malignant crypts from two patients. The absorbance was higher for normal cells compared to malignant type. Interestingly, the absorbance differences for the amide II band was not significant as it was observed with various sites in the normal crypt. Also, the doublet in the region between 1000-1100 cm$^{-1}$ was not observed for malignant cells. The significant absorbance differences were found only between 1000-1500 cm$^{-1}$, which accounts for vital metabolites of the cells.

4.3.3 Phosphate Level and Amide I/II Intensity Ratio

The phosphate content derived by integrating the area under the symmetric (980-1149 cm$^{-1}$) and asymmetric (1151-1350 cm$^{-1}$) regions using OPUS software of the phosphate group is presented in Figure 4.3.6a. Our results showed that the phosphate content at the bottom site of the crypt was higher than the top in most of the measurements. The difference in phosphate content between bottom and top was higher for young patients (Crypt Nos: 1-2 and 9-11) relative to older ones. Among
them, the difference was largest for the young patient having advanced stage of malignancy. Compared to this patient, the difference was lower for the young at the moderate stage of malignancy. But, in older patients the difference in phosphate content was not significant irrespective of the grade of the malignancy. Several reports suggest that the amide I/II intensity ratio increase with DNA content of the epithelial cells [118], whereas in the case of RBC (Red Blood Cells), the intensity ratio of amide I/II is nearly the same as any other pure protein spectrum. The light transmitted during measurement is inversely proportional to the packing density of the nucleus and it can be seen as the variation of amide I/II ratio [140]. The ratio of the integrated absorbance under amide I/II is shown in Figure 4.3.6b. The results indicated that the cells at top of the crypt had higher DNA absorption contributed to the amide I than the bottom. To confirm the above results, we performed fitting procedure for all the spectra and derived the analytical area for the phosphate and amide I regions. The results (not shown) were in perfect agreement with the above procedure. The correlation between these two metabolites was found for all the samples in this study.

![Graph showing the difference in phosphate level between top and bottom of crypts](image-url)
Figure 4.3.6: a) The phosphate content of the epithelial cells at the bottom and top of normal crypts obtained from four patients. The phosphate content was calculated as the sum of symmetric and asymmetric stretching vibration modes of the phosphate group. OPUS software was used for this purpose b) Amide I/II ratio as an indicator of DNA content in the cells. The integrated area under the absorption peaks of amide I and II were calculated after baseline correction and amide I normalization.

4.3.4. Discussion

The absorbance differences between bottom and top sites can be understood from the cellular life cycle in the normal crypt of the human intestine. Generally, in normal crypt, the epithelial cells are generated in the bottom portion and move further with differentiation and finally exit ending up in apoptosis. The recent model given by Moss et al [132] (schematically shown in Figure 4.3.1) on histogenesis of colonic adenomas claims that in adenomas, the proliferation of cells is predominant at lumen (top of the crypt) and apoptosis at the base (bottom of the crypt), a complete reversal of normal pattern. The concentration of various metabolites is higher in the cells at the bottom site compared to the top of crypt accounts for the absorbance changes between these two sites. This is the reason that the absorbance differences
between bottom and top of the crypt is reflected in the entire region of the spectrum and it may be due to the higher metabolic rate of the rapidly growing cells in the bottom relative to the ones undergoing apoptosis at the top of the crypt. This hypothesis also explains the intensity differences between normal and malignant cells from the bottom of the crypt (Figure 4.3.5). The pattern change in the phosphate region is a useful indicator of any deviation from the normal growth status of the cell. This has been observed also in the malignant cells from the human intestine. The frequency shifts observed for amide I and II between bottom and top sites of the crypts was highest for the patient who was young and having advanced stage of colon cancer. The extent of frequency shifts in the amide I peak (about four wavenumbers) was in the following order: young & advanced > old & advanced > young & moderate > old & moderate. There is a clear trend with age and grade of the malignancy. Probably the conformations associated with some of the proteins would account for these frequency shifts.

The calculation of metabolites such as phosphate and amide I/II contents provided some additional interesting insights. The difference in phosphate content between bottom and top of the crypt was largest in the young and having advanced stage of malignancy. The process of necrosis [141] leading to decreased nuclear density may be the probable reason for this observed differences in absorbance between top and bottom sites of the crypt. The young and moderate also showed a marked difference between bottom and top sites of the crypt. Other old patients did not show significant changes. The difference in amide I/II was significant for young (both advanced and moderate) and also for old who was in the advanced stage of malignancy.

Our results clearly indicate that age and grade of malignancy are the key factors affecting the epithelial cellular activity in the normal crypt leading to the observed variations in the FTIR spectra. It is important to note that the microscopic FTIR spectra of normal crypt at different sites can provide valuable information. In particular, the literature points out that the early onset of colon cancer is mainly attributed to genetic reasons [142, 143]. The limited study reported here shows the power of FTIR-MSP in understanding the various factors to be considered in the early diagnosis of malignancy using optical biopsy. More extensive studies using
FTIR-MSP may give rise to a novel tool for clinical diagnosis of familial cases with good accuracy.

4.3.5 Summary and Conclusions

The human intestine is made up of micro-structures called crypts containing epithelial cells. It is U shaped where the cells are generated in the bottom, move upward and finally shed into the lumen. In our studies, we have collected FTIR-MSP spectra of cells at the bottom (11 spectra) and top (11 spectra) of normal crypts from the biopsies of colon cancer patients. The absorbance due to the cells at the bottom was higher than at the top site of the crypt in all the cases. The higher metabolic rate of rapidly growing cells accounts for this observation. There were significant pattern changes and frequency shifts observed between cells at the top and bottom of the crypt. The cells at bottom of normal crypt showed higher absorbance than the malignant crypt. The histogenesis of normal and malignant cells is the reason for these differences in the absorbance. Our results on biological markers derived from the spectra showed that there was large difference between the bottom and top of the crypt for the patient who was young and having advanced stage of malignancy. Age and grading of malignancy are key factors affecting FTIR spectral parameters of the normal crypt. Our study may lead to a new path in the diagnosis of colon cancer due to genetic sources rather than sporadic reasons.
5) References


79. K. Miller and J.D. Waye, “Colorectal polyps in the elderly: what should be done?” , Drugs Aging, 19(6), 2002, pp.: 393-404


89. IRscope II, User’s Manual, BRUKER.

90. PeakFit manual book.


Appendix I) Refereed papers and Proceeding

Refereed Articles


**Proceeding**


# Appendix II

## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Absorbance</td>
</tr>
<tr>
<td>ADC</td>
<td>Analog Digital Converter</td>
</tr>
<tr>
<td>AgBr</td>
<td>Silver bromide</td>
</tr>
<tr>
<td>ANN</td>
<td>Artificial Neural Network</td>
</tr>
<tr>
<td>AQP</td>
<td>Acquisition processor</td>
</tr>
<tr>
<td>ATR</td>
<td>Attenuated Total Reflectance</td>
</tr>
<tr>
<td>BaF$_2$</td>
<td>Barium fluoride</td>
</tr>
<tr>
<td>Balb/3T3</td>
<td>Murine fibroblasts cell lines</td>
</tr>
<tr>
<td>BM</td>
<td>Normal rabbit bone marrow fibroblasts</td>
</tr>
<tr>
<td>BMT</td>
<td>Rabbit bone marrow fibroblasts transformed by MuSV</td>
</tr>
<tr>
<td>CA</td>
<td>Capsid</td>
</tr>
<tr>
<td>CaF$_2$</td>
<td>Calcium fluoride</td>
</tr>
<tr>
<td>CCD</td>
<td>Coupled channel detector</td>
</tr>
<tr>
<td>c-myc</td>
<td>Type of proto-oncogene</td>
</tr>
<tr>
<td>CsI</td>
<td>Cesium Iodide</td>
</tr>
<tr>
<td>CT</td>
<td>Computerized Axial Tomography</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribo Nucleic Acid</td>
</tr>
<tr>
<td>DTGS</td>
<td>Deuterated Triglycine Sulfate</td>
</tr>
<tr>
<td>f1</td>
<td>Murine fibroblasts cell (controls)</td>
</tr>
<tr>
<td>f6</td>
<td>Murine fibroblasts cell transfected by H-ras</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal Calf Serum</td>
</tr>
<tr>
<td>FFT</td>
<td>Fast Fourier Transform</td>
</tr>
<tr>
<td>ffu</td>
<td>Focus forming units</td>
</tr>
<tr>
<td>FT</td>
<td>Fourier transform</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier Transform InfraRed.</td>
</tr>
<tr>
<td>FTIR-MSP</td>
<td>Fourier Transform InfraRed microspectroscopy</td>
</tr>
<tr>
<td>FWHM</td>
<td>Full width at half maximum.</td>
</tr>
<tr>
<td>FWT</td>
<td>Fast Wavelet Transform</td>
</tr>
<tr>
<td>GADPH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GAP</td>
<td>Protein</td>
</tr>
<tr>
<td>GC</td>
<td>Gas Chromatography</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine diphosphate</td>
</tr>
<tr>
<td>GNRP</td>
<td>Guanine Nucleotide Releasing Proteins</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine Triphosphate</td>
</tr>
<tr>
<td>HeNe</td>
<td>Helium Neon Laser</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus.</td>
</tr>
<tr>
<td>H-ras</td>
<td>Type of oncogene</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>-------------</td>
<td>-------------</td>
</tr>
<tr>
<td>HTLV</td>
<td>Human T-cell leukemia viruses.</td>
</tr>
<tr>
<td>IR</td>
<td>Infra Red</td>
</tr>
<tr>
<td>kDa</td>
<td>1000 mass unit</td>
</tr>
<tr>
<td>K-ras</td>
<td>Genes</td>
</tr>
<tr>
<td>LDA</td>
<td>Linear Discriminant Analysis</td>
</tr>
<tr>
<td>MA</td>
<td>Matrix protein</td>
</tr>
<tr>
<td>MCT</td>
<td>Mercury Cadmium Tellurium (HgCdTe)</td>
</tr>
<tr>
<td>MLP</td>
<td>Multilayer Perceptron</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MuLV</td>
<td>Moloney murine leukemia virus</td>
</tr>
<tr>
<td>MuSV</td>
<td>Murine Sarcoma Virus</td>
</tr>
<tr>
<td>NBCS</td>
<td>New born calf serum</td>
</tr>
<tr>
<td>NC</td>
<td>Nucleocapsid</td>
</tr>
<tr>
<td>NIH</td>
<td>Normal mouse fibroblasts</td>
</tr>
<tr>
<td>NIH/3T3</td>
<td>Mouse fibroblast cell lines</td>
</tr>
<tr>
<td>NN</td>
<td>Neural Networks</td>
</tr>
<tr>
<td>N-ras</td>
<td>Genes</td>
</tr>
<tr>
<td>onc</td>
<td>Oncogene</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>ras</td>
<td>Families of gene types</td>
</tr>
<tr>
<td>RBC</td>
<td>Red Blood Cells</td>
</tr>
<tr>
<td>RMS</td>
<td>Root Mean Square</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribo Nucleic Acid</td>
</tr>
<tr>
<td>SiC</td>
<td>Ceramic Silicon Carbide</td>
</tr>
<tr>
<td>SNR</td>
<td>Signal to Noise Ratio</td>
</tr>
<tr>
<td>src</td>
<td>Families of gene types</td>
</tr>
<tr>
<td>SU</td>
<td>Surface glycoproteins</td>
</tr>
<tr>
<td>SUMC</td>
<td>Soroka University Medical Center</td>
</tr>
<tr>
<td>T</td>
<td>Transmittance</td>
</tr>
<tr>
<td>T24</td>
<td>Human bladder carcinoma</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transporter RNA</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
</tr>
<tr>
<td>Vis</td>
<td>Visible</td>
</tr>
<tr>
<td>ZnS</td>
<td>Zinc sulfide</td>
</tr>
<tr>
<td>ZnSe</td>
<td>Zinc Selenide</td>
</tr>
<tr>
<td>ZPD</td>
<td>Zero Path Difference</td>
</tr>
</tbody>
</table>
Linear Discriminant Analysis (LDA) was employed. The results of the spectroscopic analysis and morphological data showed a 89% correct classification rate. It was observed that there are factors of influence that exist, such as age, sex, and the grading of the tumor. The factors of influence that exist, such as age, sex, and the grading of the tumor, may lead to misclassifications. However, we were able to classify three types of tumors: normal tissues, carcinomatous tissues, and adenomatous polyps. We performed these measurements in 20 patients, and the success rates were 89, 81, and 83%, respectively.
1. תאי עבד פיברובלסטים (Balb/3T3) גורמיים (f1) העבר
פיברובלסטים שוהותר אלול המראקנוף
2. תאי חולדה פיברובלסטים (NIH3T3) גורמיים (f6)
h- ras (NIH-MuSV) MuSV
פיברובלסטים שערור תטרופומضة ריאלית עפר
3. תאי פיברובלסטים ראשוניים גורמיים (BM) ממח העבר
4. תאי פיברובלסטים ראשוניים שערור תטרופומضة (BMT) MuSV

מסקנות: ההפרדה של צורות שלקרמה המעד נורמליות צמצום בוליע, זורה
זיסת הקרקמה הפוליפית (במקורה של מעי) שלקרמה הטרט, בתוחם התקני 600-3200 cm⁻¹ בשטח קרמת נורמל של פיי 0.5 שנאי I. השינויים הפיקרולים בתאום תאיים ראשוניים
ijke צמצום של מוז diferença בין יזור גולמי זיסת הטרט של התאיים. הזווית של האור
ראשוניים ויורכ ש PARAM גלא של מחלוק במטרה רבח הזרמת לתיום "גורמיים" תטרובית
וזיוסו, וייתכן ש oran של תאיים הפיקרולים שוחב

3. תאי הופוספטים בתאמה גורמיים יזור גוללה מקרמה הפיקרולים של התאיים הפיקרולים
בכל תאי להrlen התאמה. זה ה- RNA/DNA בקרמה הפיקרולים שלו גבולה בשאלה לكرמה
גורמיים. התאורת היא נמצאת בקרמה הפיקרולים עם רמח השקפי של התאיים הפיקרולים
וזיוסו יזור בתאיים הפיקרולים.
הקשר והזקן

בזכותのではないか, חולק ביד קובע המאוזHDR
והם תלמידים, בסיסי להבנה בתוכן האינפרארADX
שופטים בהצלחה של מולקולות מתוכנות או מעין "שיפוץ" בודגיית
עבורה. הבוליש התאורטי מתנהל עם בתוכן האינפרארADX
האמצויות
בתחום הספקטרום 25-3 מטרים

スペクトル האינפרא-אדום דווקא בריגוד שכרכלים הכוכבים של השמש, ש المختلفة
המכנה המולטיקולרי, הוא מפשיסים ממידות גם בטריטוין המולטיקולריים מוכרים. כמו
スペクトרום פיקוק-אינפרא-אדום הפרספקטתו האינפרא-אדום, חפץ לשיטות לאליסטים
הביץונאוג'ה, שיכולים להיבדיל מספר שונים ממיקורים-אורוג'יו, אך גם מהדידים
מצעת היחסית, רוגר רכמתו של שוג. נית לולאה אחר השניות המבניות
ברמן התיאורית והнт-תאראות אחר מפתיתים ברקמה האנברנילית, שיכולים את רצב
הצורות של טריפ, עיון מידה ההבדל במשבר תוכנות אופטיסות שיןית לולאה אוות
בעורスペクトרום פיקוק-אינפרא-אדום. אופני וייבטפי המולטיקולריים שופעים
スペクトרום使って האינפרא-אדום, ממאטיאס את ההכרבה הבוכנמי של התאום
והמטיבים

המשטח העקרורי של עבורה, והן להעריך את הפוטנציאל שלĠすぎונאוג'ה
השושה של מיקרו-スペクトרום פיקוק-אינפרא-אדום, בתוכן הפרואה
ולוחותفق עבורה קדמתי. עבורה והכפייה שלשוף שגין שגין של תאי
ברבון. כימי קיים ממית רכמתו לע מייל להדריך את הפוטנציאל של השישת
להבדיל בין שולשות הסוגים השונים של הרקמה: נורמלואוט, פיר-סרטיון (וליוס)

ורקטרניות.

ארבעה המערכים שמדוניו עבורה

(FTIR-MS) נ

(2)
תקציר

להקמת המאמרים הרבים המושקעים הבחנה מחלה סרטן, מונע זיווג בול, הטרון
עדין נושב לורא מספר את בדואים המותעות. מחלה סרטן ומורמת לכלכל
וחתלת, אף כעל המותעה. חטול על המידה הווה במע נוספים (איברח, תרופת,
ningarיות וו...), ובינוקפין (חטול ככדי מחלה התחל, או חטול מסיס עיון התחל,
הפסק ככדי חתנצאה ממוות ברום עד מ...). ניטפ ש�新ון התיעולה ביוור להרדה
רמט התראומה האישית והטל الكلכל על התחל ומידיה, היא בשמיתו כמקדם
וומידיה עם התחל. איברח ממדים וומידיה עם התחל ואת התערובת המשר
התחל עד מצלבני האינטניפים, בשתי פוחת אוגניטיב, וככ ממדילו אצ סכני
התחל לחולות.

ש伊拉 את הנח 마련 מחלה הסטרן הקימומית הים כלולות: (א) הבנת נוגידו
ברקמות (ב) (ב) (מישובה המגננת ורגימית), בידיקת
 setPosition(ב) (ברקמות) המיקורסנופית של הרקמה או עם הרקמה (קובוט, חיתוך, וצבעה) לפל
האנלני, או יכל גלול החינוות חנצה מחולות הדרים בהסעה של האיס
סרטינים וסמס סטרטינים, בידיקת. הבדיקת הפיסית של התחלים היא סובייקטיבית
מטבעה כוין שינה חלולים בנים ושנים מצורים bachד. שיותה- (א) מודר
חיית שינה איבחר ויתורית לבידיקת חולות.

 seçenטסוספוזיטי אינפרא-אדום (FTIR)ディון יחותינית בשילוח לא פלשית.
לאופטי המבנה והוביליציוני של חומרים שלím. קיימים הרבח ספרים על דוגמה של
המבה של השלכות המבנה של חלבונים, ברמודת אינפיטיב, בימוקולוזה שקול עי

שם ב- FTIR
העבורהنشرתהבהורכת: פרופסור שאול מרדבר

במחלקה: פיזיקה

בפקולטה: מדעי הטבע
шимוים בויניקר-ספרטסקטופייא איינפרא אדום
ונייטוט יᡥושב מתקדמת לאפית רכמת Mayıs של
אדם, והאוליגום וסרונימי בחרבון.

מ hakkְל לש מִלְיִי חקק של הדרישות לבָּלוּת הוהא"זוקטרז המֶלְּסִפִּיָה"

מאחט

אוחם סלמאן

ווחש לשוגג או תגבורשיות ב נוירוג' גגב

________________________
אנישור המנהלה:

________________________
אנישור דוק מתי הספַר ללוימו מ hakkְל מתקדמים:

"ל' אדור אי-הס"ז"-

פּברואר-2003

בואר שבע
шимוש במא過程中-سفכותרוסקופית אין фа אדום
וישتصريح היות מתקדים אלא יי רקמת עלי של
אדם, והא всяк高尔גד וטרכניום חרבון.

מחקער לשם מילוי חלקי של הדרישה לפי הבול "דוקטור לפילוסופיה"

מאח

אמות סטמאר

והנה להסניאט אוניברסיטת בן גוריון בנגב

גל- אדר א-תט"ש 2003

בואר שבתא