

SPECTRALYZER: A COMPREHENSIVE PROGRAM TO CLASSIFY FTIR MICROSCOPIC DATA APPLIED FOR EARLY DETECTION OF CRITICAL AILMENTS

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Abstract: *Micro spectroscopy can be used for the early diagnosis of critical ailments. Early diagnosis is crucial for identifying the presence of a disease before it progresses beyond a critical stage. It has been shown that micro spectroscopy can be used as a non-invasive or limited-invasive approach for detecting different stages of cervical intraepithelial neoplasia.. The unique spectral "fingerprint" that characterizes premalignant cells can be used to differentiate each stage from normal (healthy) cells. Techniques lacking automatic, objective, sensitive and rapid diagnostic tools are not sufficient. We have developed SPECTRALYZER, a novel micro spectroscopical computational tool for automated complementary diagnostic analysis.*

Key words: *Micro spectroscopy; computational tool; SPECTRALYZER*

1. Background

Cervical cancer especially invasive squamous cell carcinoma is the second most prevalent cancer among women [1-4]⁴ and squamous cell carcinoma constitutes 80–90% of cervical cancers. Screening programs, especially the identification of precancerous lesions at an early stage, can produce good prognoses and prompt early treatment to prevent advanced-stage cancer and death [5]

Current tests, such as PAP smears and a polymerase chain reaction [6], though they can detect cervical cancer, do not give the total diagnosis unless they are supported by histology or results of biopsies.

During the onset of cervical cancer and neoplasia, the biochemical composition of tissues becomes altered [7]. Several approaches to quantitatively link these changes with spectral characteristics [8, 9] involve sophisticated cell mechanisms and techniques to interpret them. Fourier-transform infrared micro spectroscopy (FTIR-MSP) has been shown to be a promising diagnostic tool in monitoring biochemical changes in cells during experiments with exfoliated cervical cells and biopsies [10]. Materials with differing chemical compositions or structures will exhibit different absorption spectra, which can be used as "fingerprints" for the characterization of these materials [11], and thus the biochemical changes between neoplastic stages can be used to identify them.

2. Program

THE SPECTRALYZER was developed in order to automate and facilitate the Fourier-Transform Infrared (FTIR) analysis on biological samples. Many spectroscopic tools can generate a text-based list of IR spectral data, i.e. coordinate points representing absorbance as a function of wavenumber, that can be saved as simple text files Investigation Spectroscopy in the mid-IR spectral range includes analysis and comparison between many samples and between the sample and known markers to identify the unique characteristics of the sample which might reveal the unique substance found in one sample but not the other. The idea beyond the developing of the SPECTRALYZER is to build an automatic, objective, sensitive and rapid analyze tool that would designated for early detection of biological spectroscopy. The SPECTRALYZER can easily read multiple spectra files for fast and accurate scrutiny and comparison. Spectra may be manipulated and analyzed by this program in many different ways: subtracted, shifted, scaled, and "smoothed", as well as matching peaks, principal component, and linear discriminant analysis.

A relational database development was required to process this vast information. Such a database allows further analyses to discover new substances and to collect biological knowledge of the studied sample(s). This task required the use of several bioinformatics software tools and database searches, executed in a batch mode and often required additional programming.

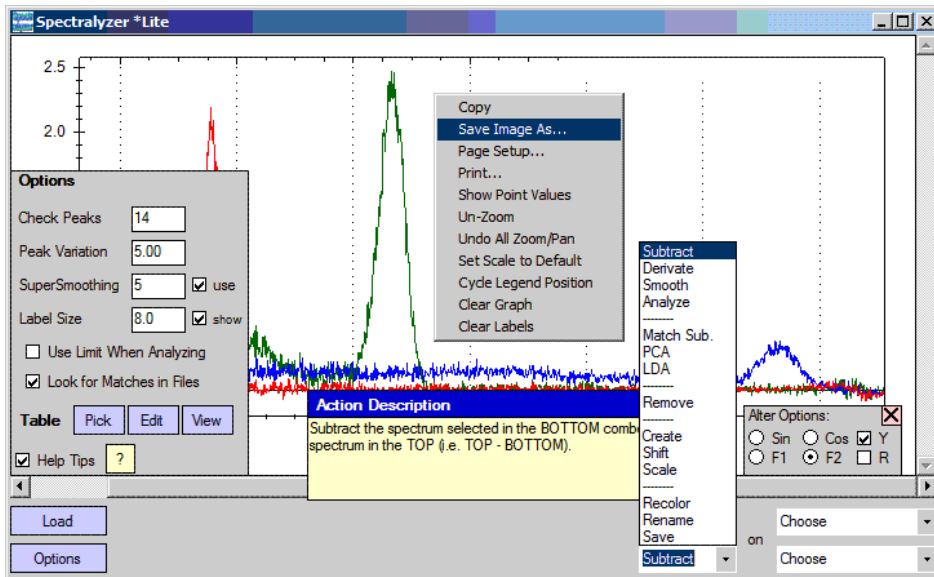


Figure 1. SPECTRALYZER main window with sample spectra and Options, Alter, and Right-Click menus shown

The program window is divided into two main sections -- the graphical pane and the toolbar (Figure 1.). The toolbar, which contains action buttons and selection boxes, is located along the bottom of the window to allocate the majority of the window area for the graphical pane located above. There are two selection boxes (combo boxes) for choosing the spectrum and the action to be performed. Buttons will load spectra, show the options menu, or perform the selected action.

Two additional information panes, normally hidden, will let users view or change some of the program settings or alter the selected spectrum according to the current action, respectively. There are also popup dialogs reporting useful information about each button or selected action.

3. Technical Background and Software Development

The SPECTRALYZER was then constructed in C#, using Microsoft Visual Studio .NET 2003 [12]. The SPECTRALYZER program was developed under the requirements and conditions of Object Oriented Analysis Design and Programming [13- 16] with particular paid attention to form techniques such as UML (Unified Modeling Language) diagramming [17-19] and design patterns [20, 21]. The use of iterative design, parallel development, and high modularity all enhance maintainability, ease of verification, and code reusability.

The SPECTRALYZER program has been developed as a two iteration process of three steps each: alpha, beta, and release. The first iteration was a module serving as an independent confirmation tool. After a Software Test Plan, Description, and Report according to MIL-STD-498 [22], the program was moved to C# under Visual Studio .NET 2005 in the next iteration in order to more easily build a user interface. At the second iteration the SPECTRALYZER passed the alpha stage; having undergone the major testing phases (requirements, design, program, and installation) [23, 24], it was reviewed under rigorous real-life conditions by typical users in the beta step. User feedback, suggestions, and accuracy assessments were incorporated into the program, which was subjected to

regression testing to make sure none of the improvements cause earlier passed tests to fail. After success it was published as the release version.

4. Advantages of SPECTRALYZER

The SPECTRALYZER program contains many of the same abilities as commercial products like Bruker Optic's OPUS, such as easy spectrum manipulation, saving spectral graphs for printing, and spectra library management, yet it comes in a package hundreds of times smaller. SPECTRALYZER was created specifically for analyzing photonic spectra, especially those from the FTIR spectroscopy of biological samples. It is not enough to use one method to find the markers for various ailments. This program has the ability to readily use multiple methods for analyzing different spectra, improving the chances of accurately detecting the important markers.

These methods include spectrum comparison, principal-component analysis (PCA) [25], and linear-discriminant analysis (LDA) [26, 27]. For discovering component elements (represented by specific peaks in a spectrum), the user can build, arrange, and update their own database which is used for peak matching. Scaling a spectrum by a ratio, such as DNA/RNA ratio (1121/1020 cm^{-1}) [28], is a simple action. All of the functions of the program are tightly integrated into a straightforward and consistent interface. Many of the default or underlying system options are easily changed in the settings file, and the entire program is ready for localization to most languages just by changing a single text file -- both of which are fully explained within their respective files. The entire program requires only a few files to begin, and is ready to run on a Windows with .NET computer without any installation -- thus the program could be loaded on a disk and transferred between workstations anywhere. The SPECTRALYZER, implement a "protocol macro" function, whereby automated multiple actions may be combined and saved for rapid and easier repetition.

6. Sample preparation

The method of Argov et al. [29] was followed for sample preparation. Biopsy samples were taken from several stages of neoplastic cells -- "normal" (healthy) cells, "CIN1" stage is mildly dysplastic, moderately dysplastic stage "CIN2", a severe dysplasia is "CIN3" cases (which is considered as cancer *in situ*), and an "invasive" stage that has progressed beyond CIN3.

Two adjacent paraffin sections were cut from each biopsy; one was placed on a zinc-selenium slide and the other on glass slide. Care was taken to ensure that tissue sections were practically identical with a thickness of 10 μm . 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 parallel histology review. In cases where the biopsy showed histological similarity to more than one neoplastic stage, the different regions were identified and measured separately with the help of an expert pathologist who distinguished the stages under microscope.

The microscopic sites of measurements were taken keeping in view the occurrence and diagnostic features of the neoplasia in relation to its staging as described [30,31] To achieve high signal to noise ratio (SNR) 256 co-added scans were collected in each

measurement in the wavenumber region 600 to 4000 cm^{-1} . The spectra were baseline corrected automatically by the sensing equipment using OPUS software. The spectra were normalized to the amide I (1652 cm^{-1}) absorbance peak for calculations and subsequent analyses.

7. Analysis demonstration

An FTIR spectrum was selected from each cervical cancer stage to demonstrate how the SPECTRALYZER program may be used. Opening the program immediately requests a spectrum file to load, and selecting the cervical spectrum file loads all five distinctive spectra as seen in Figure 2.

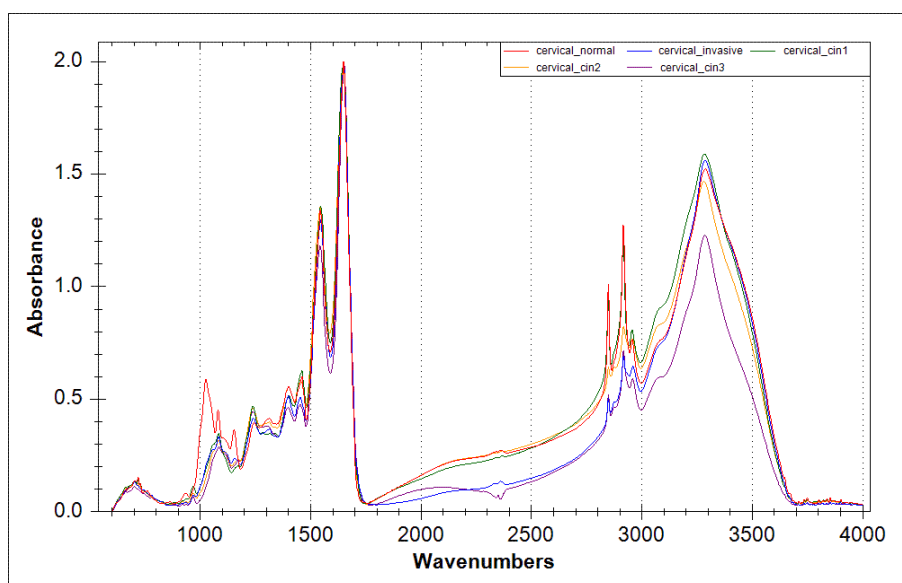


Figure 2. Selected spectra of various stages of cervical neoplasia (normal, CIN1, CIN2, CIN3 and invasive)

The first and simplest step towards demonstrating the differences between stages would be to subtract each spectrum from the known "healthy" section. In the action selection box, we choose 'Subtract', and then following the directions in the Action Description box we select the "normal" spectrum at the top spectrum selection and each of the other stages at the bottom spectrum selection before pressing the 'Perform' button. This produces the result seen in Figure 3. We can begin to see the differences between stages here, mainly between 1700 - 2800 and 3000 - 3600 cm^{-1} wavenumbers, but defining characteristics of each stage are not clear yet.

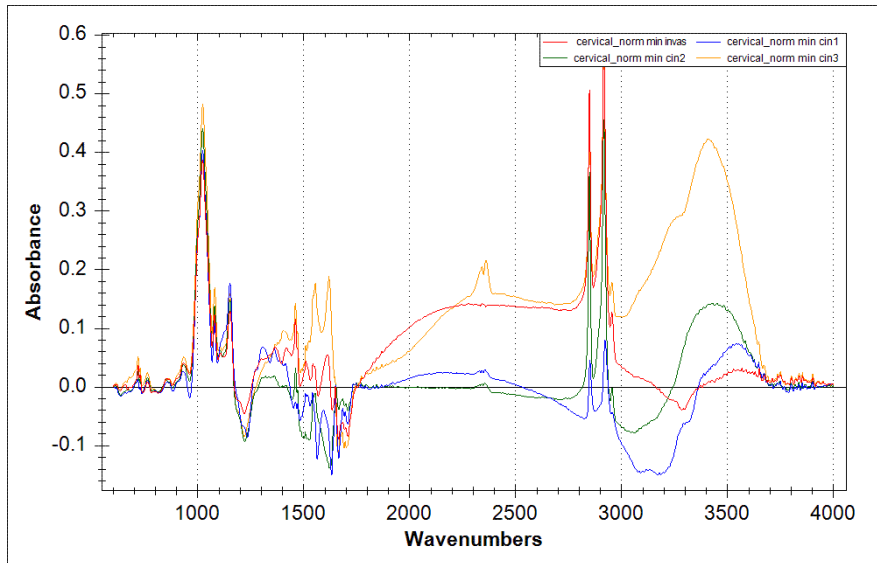


Figure 3. Comparison between the difference spectra (between normal and invasive, CIN1, CIN2, or CIN3)

We next perform a Principle Component Analysis (PCA) on each of the spectra (normal, invasive, CIN1 - 3). As mentioned earlier with the 'Subtract' action, we follow similar steps to select 'PCA' from the action list, and following the Action Description we select each spectrum in the top spectrum selection box, then press 'Perform'. We are then prompted to select from among the eigenvalue/eigenvector pairs calculated by the PCA Figure 4. After selecting both, a new transformed 'spectrum' is generated and added to the plot with the suffix "pca". Our new coordinate axes represent the variation along the chosen eigenvectors, which shows the significance of each original datum. We repeat this with each of the other base spectra to end up with five new transformed spectra. However, while we can still see some differences between them, it is not much more informative than the original spectra. When we subtract each suspicious PCA "spectrum" from the normal, the differences become more clear (Figure 5.), and we can use their positions and alignments to help determine their stage (class). At the low end of the PCA "scale" (negative x-values), we see that advanced stages of CIN are higher on the y-axis (with the exception of the invasive neoplasia), while on the positive side we see that the advanced stages appear lower.

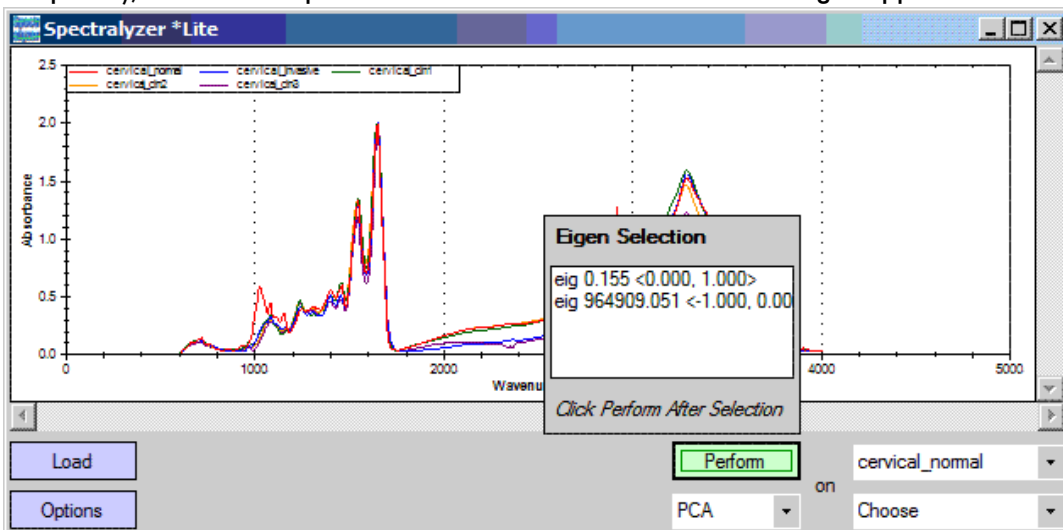


Figure 4. Performing the Principle Component Analysis (PCA) - selecting eigenvalues/eigenvectors

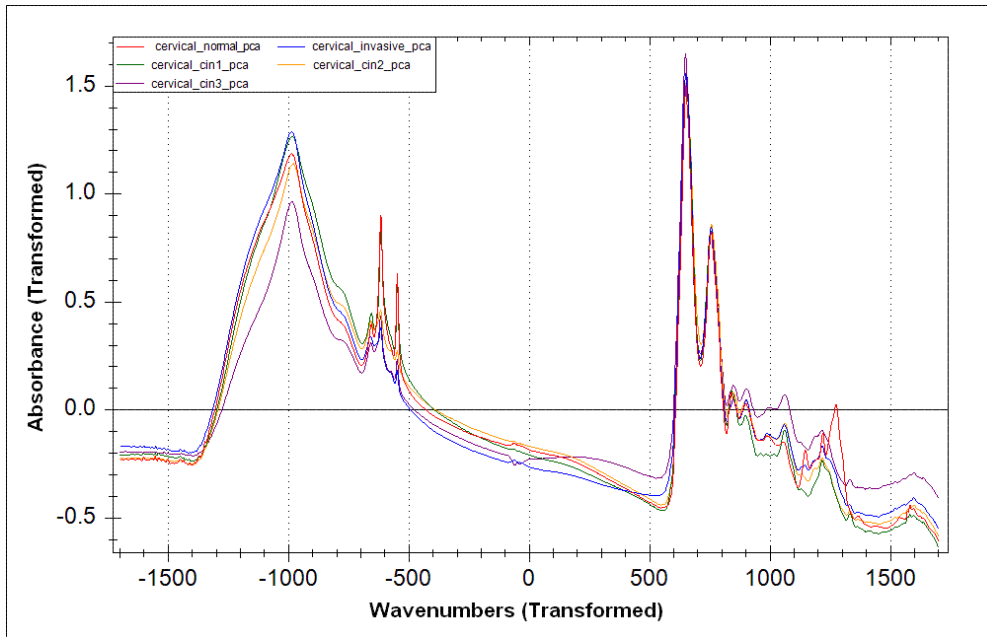


Figure 5. Spectra transformed by the Principle Component Analysis (PCA). We note that the coordinate space has also been transformed.

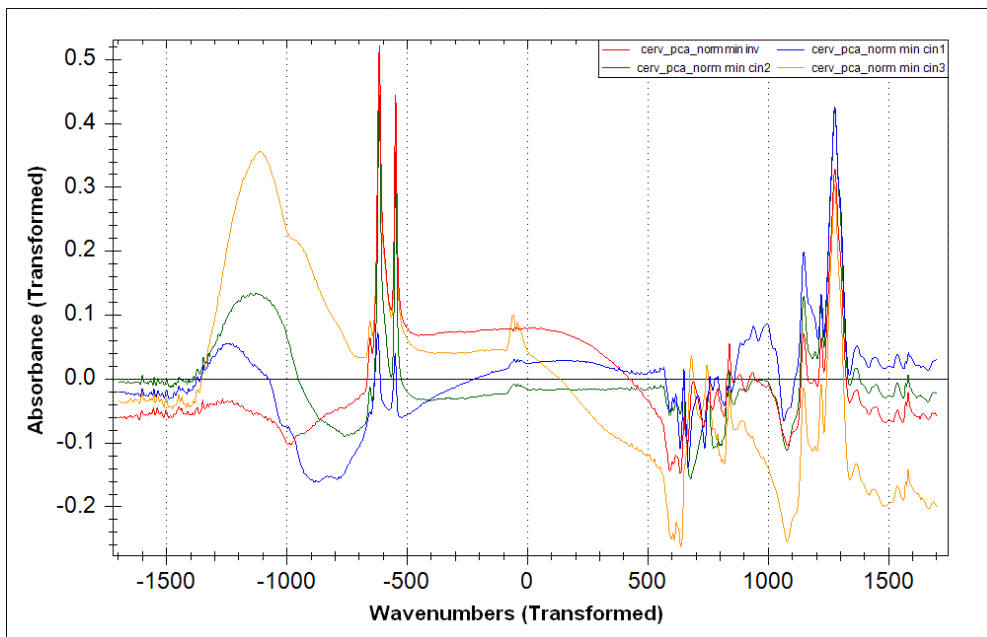


Figure 6. Differences PCA spectra for each stage with respect to normal. The wavenumber axes has been transformed

We can further differentiate the spectra using Linear Discriminant Analysis. Again, as easily as before, we select the action 'LDA' and follow the instructions (Figure 7); performing the analysis on each stage (bottom selection) with respect to the "normal" spectrum (top selection).

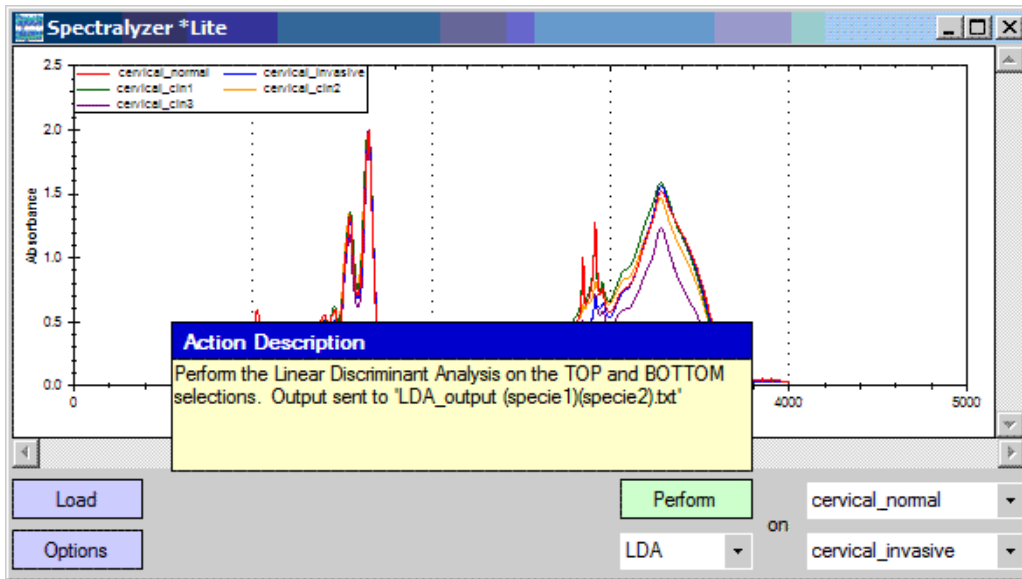


Figure 7. Performing Linear Discriminant Analysis (LDA) -- instructions shown, before saving the analysis file

LDA creates three new spectra for each operation -- the transform of each original spectrum, and the threshold between the two. The new coordinate axes represent how the "object feature" (X) determines the "categorized group" (Y) based on the original axes. The results of the LDA are automatically saved in a text file of the form "LDA output (specie1)(specie2).txt", containing the new coordinates and their "reclassification" as well as a confusion table describing the accuracy of the LDA predictions. When we examine the transformed groupings, we can immediately see a gradual difference in the progression of the neoplasia (Figure 8). From the LDA, there are three distinct areas of the transformed spectra -- a lower region between 0 and -1, the middle region between 0 and 4, and an upper section above 4. As the neoplasia advances from normal to CIN3, the three regions show increasing amplitude around their respective threshold lines, but when it progress to the invasive stage the amplitudes decrease slightly.

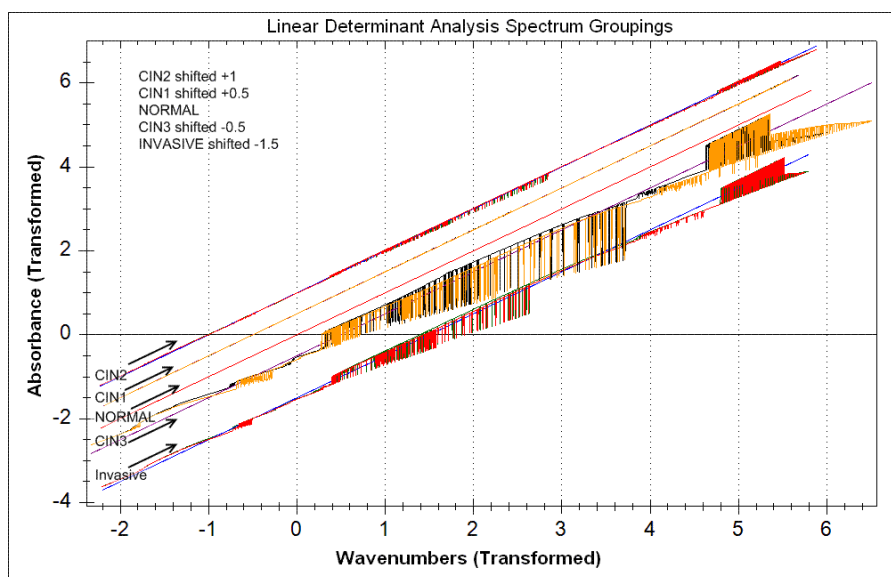


Figure 8. Transformed "spectra" from the LDA; spectra are shifted arbitrarily as indicated in the figure to improve visibility. The wavenumbers axes is transformed

8. Conclusion

The PAP smear, which is currently used for initial screening for cervical cancer, has the major drawback of false negatives, and its analysis is time consuming. Thus other methods that are objective, rapid, and less prone to error are desirable. Changes in biochemical compositions can be an indication of cancer [32,33]. However, such studies are expensive and complicated [34]. Earlier research highlights the importance of detection of biochemical changes in formalin-fixed tissue for diagnosis of cervical cancer through the use of Fourier-transform infrared micro spectroscopy. The changes manifested in the FTIR spectra were used as fingerprints with which to classify and diagnose the various types and stages of disease that are not easily detectable through conventional methods.

Thus approved techniques without an automatic, objective, sensitive and rapid analyze tool are not sufficient, and it is for this reason that the SPECTRALYZER was developed as a complementary. In effect it constitutes a tool that melds traditional analysis methods with advanced computational methods such as the Principal Component and the Linear Discriminant Analyses which are comparable to the gold standard approach.

In Cervical Cancer diagnosis test case, the meld of the Principal Component and the Linear Discriminant Analyses performed by the SPECTRALYZER program exhibit promising potential for an earlier, more accurate diagnosis of cervical neoplasia, and should certainly be investigated further. For other ailments with biochemical changes, accurate microscopic FTIR data in tandem with the SPECTRALYZER program may lead to earlier detection, critical for identifying these conditions before they become untreatable.

9. Downloading and installing SPECTRALYZER

News, updates, and all versions of SPECTRALYZER are all available from our web site : <http://nmcrc.sce.ac.il>.

To install SPECTRALYZER, the compressed file can be downloaded from the NMCRC website. It needs only to be unzipped to a folder of the user's choice, and can be run immediately by running the executable file.

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