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Comparative Study of Small Size Tumors using a Home-made Confocal Laser Scanning System and Spectroscopic Techniques

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Abstract* - Biomedical optics is a fast growing field of research. The development of a technique that is less invasive and provides real time and quantitative information about tissue biochemistry is of great importance for cancer diagnosis. Microscopy enables the scientist to see and elucidate special features and capabilities that allow different cell types to thrive in different environments and to perform different functions. One of the greatest advances in light microscopy within the past two decades is the development of confocal laser scanning microscope system (CLSMS) which is a relatively new light microscopical imaging technique, and is considered a very successful tool that has greatly impacted biological research and became a valuable tool in many fields of study, due to its superior axial and lateral resolution over wide-field imaging but it is a very expensive tool. The work presented in this thesis is intended to serve as a primer for basic confocal microscopy theory as well as a description of a low-cost method of design and construction of a home-made confocal laser scanning microscopy system. We have provided a simple and affordable method for building a confocal scanning laser microscopy system on a modest budget with decent lateral resolution and 2D dissection capability comparable to that of commercial systems. The digital images obtained as the results of our system (AY128R) CLSMS are compared to the digital images from the commercial CLSMS ,which is about 80% similar to original image the structure of the neoplasia cells and malignant cells are appear. Physical and optical comparative study between two optical techniques (microscopic and spectroscopic) techniques with previous study for FT-Raman and FTIR spectroscopic techniques on IDC of breast samples was illustrated to determine the characterized features, advantage, similarities and differences for each optical techniques for early diagnosis breast cancer tissues.Both techniques are widely explored for analysis of tissue and cells and ultimately biomedical applications. Comparison of the spectroscopic and microscopic techniques demonstrates clear differences between the performances of two methods. Two techniques are complementary with each other. The combination of an optical microscopy and spectroscopy leads to the development of a new and powerful analytical characterization technology.

Keywords—Biomedical Optics; Laser Microscopy; CLSMS; Spectroscopic Techniques; Microscopic Techniques; Matlab

I. INTRODUCTION

Early diagnosis of cancerous tumors in its initial stage of growth is considered among the most common problems in medicine in the present days. Early detection and diagnosis can significantly reduce the risk associated with cancer and early treatment can slow or even reverse its development [1] .The change of normal tissue into a cancerous lesion is a slow process which involves alterations in the molecular level which, in later stages, alter the morphology and tissue architecture [2]. Cancer is the second leading cause of death in the world . With current technology, the time between one set of disease and detection of cancer is often ten or more years. Since cancer becomes more difficult to treat as it develops, it is crucial to detect and diagnose the disease as early as possible [3].

Traditional biopsy methods for diagnosis of cancer and other diseases are surgically invasive and often require a significant turnaround time for results. Often the patient will have to return for additional biopsies. There is also the risk that the specific location of the disease may be overlooked due to the inherent sampling nature of the procedure. Regular screening of easily accessible cancers such as those of the gastrointestinal track is rarely performed due in part to the invasiveness of traditional biopsy procedures. Truly non-invasive imaging modalities such as MRI and CT have the ability to image the entire body but do not have the resolution required to visualize the morphological and cellular changes of early stage cancer and pre-cancer.

Optical biopsy is a relatively new field that strives to provide the physician with real-time cellular imaging from a minimally invasive endoscopic procedure. Conventional microscopy has limited utility when viewing thick biological tissues. Light returned from out-of-focus regions in the sample overlaps the in-plane information causing a significant reduction in image quality. Confocal microscopes [4-6] are commonly used to investigate thick excised biological tissue samples because these instruments reject light from out-of-focus planes and provide high-quality images from within a localized region of the tissue. They are typically able to capture high-resolution images from a specific plane in the tissue within a few hundred microns of the surface. Since the majority of cancers are epithelial in nature, the confocal microscope is an excellent choice for optical biopsy. Confocal microscopes have been adapted for in-vivo imaging of skin [7-8], cornea [9-10], teeth [11], and cervix [12]. Specialized endoscopic systems have been developed for imaging deeper within the body based on VCSEL arrays [13], micromachined scan mirrors [14], single optical fibers [15,16], and fiber-optic bundles [17,18].

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II. METHODOLOGY

Design and Construction of a Homemade Confocal Laser Scanning Microscope System (CLSMS) with AY128R-Controller

Confocal scanning laser microscope is one of the most popular and widely used imaging techniques in biology. High sensitivity, spatial resolution and optical sectioning capability make it an ideal tool in the study of biological systems such as membranes, tissues and cells..However, most of the commercial confocal scanning laser microscope designs are not versatile enough to be employed in different laboratory configurations or as an educational tool for students or researches. Furthermore, commercially available confocal systems are usually too expensive for most individual researchers and very difficult for users to modify it based on their own research applications.The lack of flexibility and high cost of commercial systems have prompted many laboratories to construct their own confocal microscopes optimized for specific requirements .Here we report on our design of a simple and flexible confocal microscope (AY128R-controller) that can be used for research as well as for teaching and training.

Figure 0-1 Our complete confocal microscopy system CLSM with AY128-R microcontroller.This system is composed of inverted microscope, confocal part including scan head, laser optics, and computer.

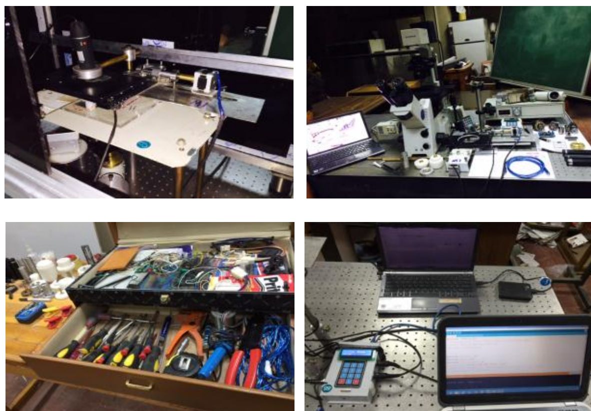

Figure 0-2 Pictures for mechanical layout of the microscope and interfacing with two computers and all tools - components in our lab in NILES to design setup for CLSM

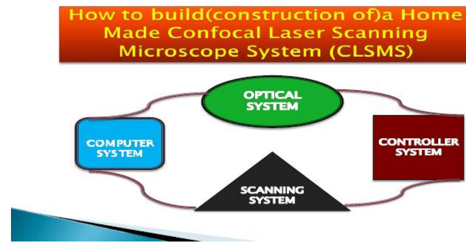

Figure 0-3 Components of a home made confocal laser scanning microscope system (CLSMS)

The design implemented in this study consists of four different systems as shown in the previous figure:

1. Optical System which includes laser, dichroic mirror, photomultiplier detector objective and inverted microscope
2. Controller System which is a control scanning stage with stepper motors, microcontroller, analog to digital converter, and electronics parts
3. Scanning System which includes interfacing mechanism between optical and controller systems
4. Computer System which includes different computer programmable and graphical user interface and image processing techniques

A. Optical System

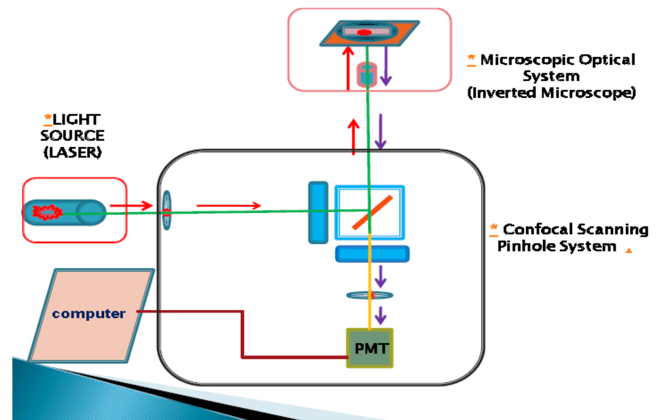

Figure 0-4 The light path of Laser-Scanning microscope set up

The optical system of CLSM include:

1. Optical bench
2. Inverted microscope
3. Objective lense
4. Illumination source (laser)
5. Motorized XY Stage
6. Dichroic mirror
7. Filter device (Pinholes)
8. Detector

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Figure 0-5 Brief detail for mechanical layout of laser beam and setup for our design

Figure 0-6 Pictures of the hard ware and layout of set up for all trial designs in all the period of the project

Inverted microscope, laser and optics which are normally available in (NILES) laboratory, were used in construction. An X–Y motorized stage , is used to raster scan the sample and construct digital images.

B. Controller System

AY128R- Controller System (Open source)

A board base on the Atmel microcontroller ATmega2560 was used with the aid of open source hardware (OHL).

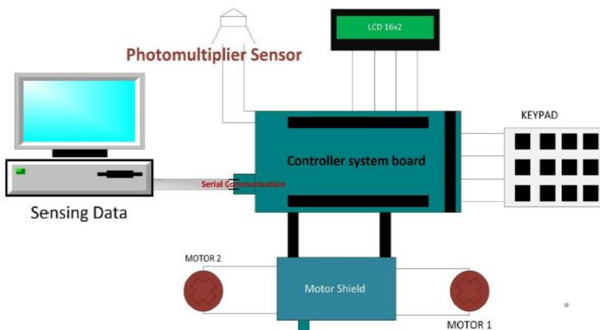

Figure 0-7 Block diagram Experimental set-up of the confocal laser system with AY128R Controller System

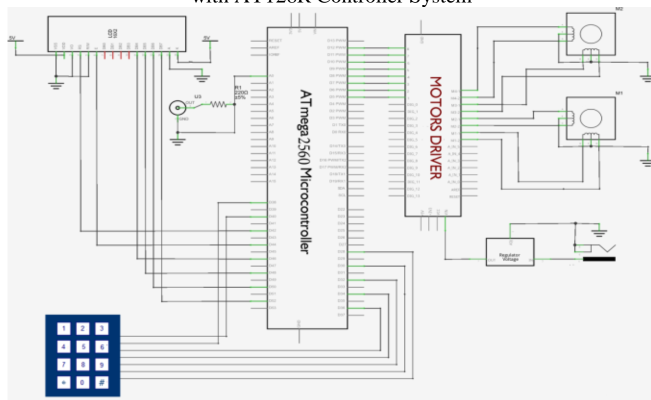

Figure 0-8 Block diagram of AY128R-controller system

Summary of the controller system:

Microcontroller	A Tmega2560
Operating Voltage	5V
Input Voltage	7-12V
Digital I/O Pins	54 (15 provide PWM output)
Analog Input Pins	16
DC Current per I/O Pin	40 mA
Flash Memory	256 KB (8 KB used by bootloader)
SRAM	8 KB
EEPROM	4 KB
Clock Speed	16 MHz

Figure 0-9 Electronic circuit for AY128R-Controller

C. Scanning System

How the system works:

1. Read sensor value
2. Convert data from analog to digital
3. Send data to computer through serial communication
4. Store data

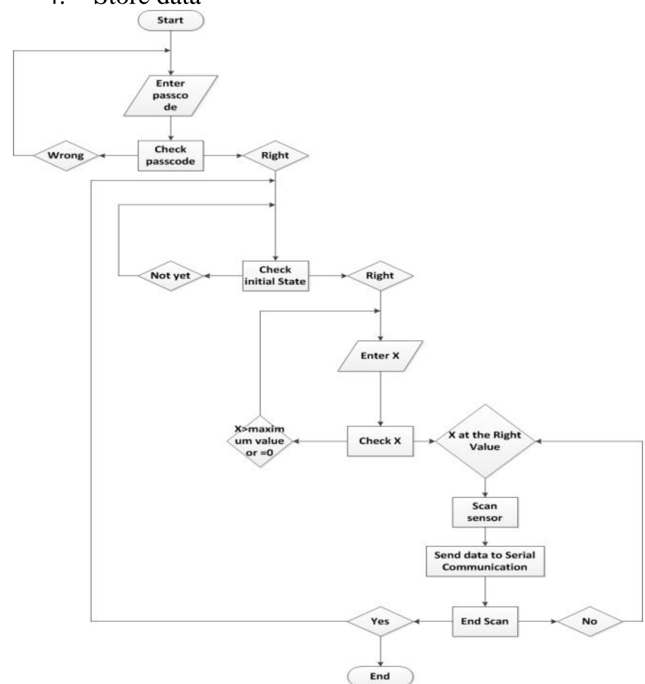

Figure 0-10 Flow chart of steps of the program

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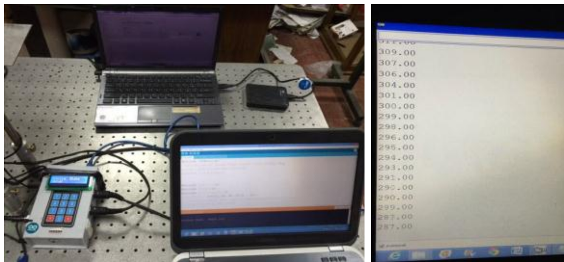

Figure 0-11 Interfacing between computer and controller system and Digital data aqcusition on the screen

D. Computer System

Several functions of the microscope are computer controlled, either via commercially available software or by homemade programs developed during the work for the present dissertation. This section describes how the computer control of the different functions is organized. Scanning and confocal data acquisition control The operation of the motorized stage, as well as the data acquisition for all types of imaging, is accomplished with the AD/DA converter. The AD/DA is computer controlled with a homemade software that consists of three parts. The first part consists of routines that operate at the lowest level, directly on the local CPU and memory of the AD/DA converter and perform the basic operations, such as scanning a line or counting photons at one pixel. These routines were programmed in a specific computer languag provided by the AD/DA manufacturer.

The second part is the PC user interface. With this software, the user can set the scanning parameters, as well as observe on-line the collected data and save it in an appropriate format. The PC user interface was programmed

Finally, the third part consists of a set of routines required for the communication between the PC user interface and the local CPU of the AD/DA converter. These routines were all programmed in matlab.

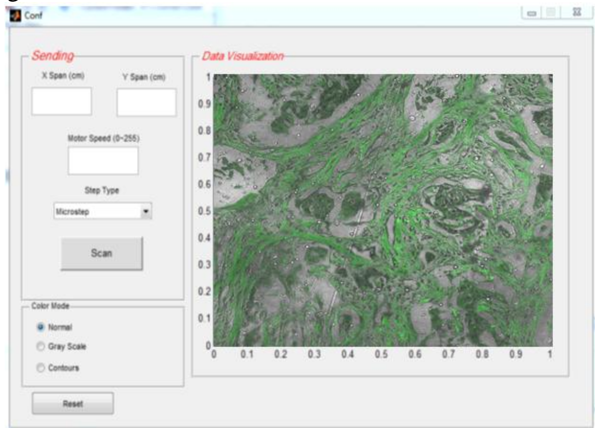

Figure 0-12 panel of the Matlab software used for data acquisition along with the image of breast tissue samples

The software allows for control of different parameters such as speed of motor step type or pixel size ,scan control scan speed .the preliminary setup acts as asystem for 2-D imaging The system is configured to move syage scanner in X-Y direction as shown in fig over various points of the object in a controller manner .at each point the reflected (or fluorescence) light which reacts the input path is scanned and detected .this intensity value is then input to the computer and recorded corresponded to its relative location on the object.

First we fill in the X-span and Y-span with the dimensions of the sample , then fill in the speed at which motors will operate on , then choose the step type which is almost microstep , then press scan. Signals will be sent to micro controller using USB cable in order to manage the motor movement by adjusting the stepper motor steps and speed . After scan is complete aplot will be presented in the axes2 area which discripes the analog data red from the sample , this image will be displayed in the form of 3 states 1-Normal (default)2- Grey scale 3-Contours Software interfacing and control of the optical scanner the Matlab _ Math Work software was used for interfacing and control of scanner motorizwd stage and acquisition of data from photomultiplier tube .to develop appropriate electric digital imaging the software for the successful implementation of confocal microscopy produced . Many open source software are available that can be custom-integrated for c the system We have written a matlab code (Math -Work) for data aqusitions and image construct which the digital date saved in excel sheet program and open source code software program for controller system The software controls the motorized stage with stepper motor and by by providing necessary voltages and acquires the voltage output of the detector. The program is divided into four modules:

Module 1 is responsible for system initialization.

Module 2 is used for generation of X–Y scanner

Module 3 is used for postprocessing of scanned images and saving the scanned image digital data in excel sheet

Module 4 is to front panel of the GUI Matlab software used for data and image acquisition along with the images of 80 biological tissue samples slice

Store data

Using a software to reading data throgh Serial communication and store it in an excel sheet.

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III. RESULTS AND DISCUSSION

The thesis is about building a homemade CLSMS with AY128R controller system and the comparative study between different" optical techniques" (microscopic imaging technique" CLSM" and different spectroscopic techniques) for early diagnosis cancer tissues.

The aim of the work is divided into two sections:

The First Section: Is a design and construction of a home-made microscopic optical technique CLSMS and digitizing an optical image which has an information about tissue structures.

The Second Section: Is about comparative study between optical imaging microscopic technique CLSMS and the previous results from different spectroscopic techniques such as FT-Raman ,FT-IR, Photoacoustic, and Flourescence Spectroscopic to illustrate their characterization Aand different features ,advantages which can differentiate between malignant cells and normal cells for early diagnosis breast cancer.

A. Section One

The confocal microscope used in this study was a home made CLSM AY 128R SYSTEM. This instrument is characterized by an inverted microscope setup connected to the computer software. The images were scanned in two dimensions along x and y axes by using a diode laser illumination at a wavelength of 532 nm wavelength with a 0.9 numerical aperture water immersion objective lens. Confocal microscopy is an emerging tool that may address the limitations of current histologic approaches by providing images of tissue architecture and morphology with subcellular resolution in real time without the need for extensive tissue processing. results from these studies demonstrate that cconfocal microscopy give images with morphologic detail that is visually comparable with that of histologic sections. Confocal microscopy has been used mainly in research laboratories; however, its application in clinical settings has been also reported.the CLSM can used of study protein –protein interaction as it provde information about the molecular structure with high rmagnifigation and powerful resolution than conventional microscopy ,live cell imaging ,study cell organelles and the physiological processes in cell as dynamics (diffusion). regarding the accuracy of our confocal microscopy system it is the color contrast between the normal fibrous tissue and the malignant fibrous tissue was not clear when compared with the original; ,confocal microscopy in which non neoplastic (normal cells) was stained red while staind dark brown the distinguee between malignant tissue and normal was obvious but the picture can illustrate the same morphological structure of the tissue and by development in the software program and in interfacing between all parts of thes seystem we can obtain an image with contrast much better than above picture.

Table 0-1 Comparison between images from inverted light microscope, from confocal imges from ziess and confocal AY128R system from H&Estained section of a region of the specimens IDC-breast tissues

Type	CLSM AY128R	CLSM Zeiss	Light microscope	No.
IDC				1
IDC				2
Normal				3

B. Section Two

Physical comparison and illustrated features and criteria for each optical methods as shown in the following tables.

Table 0-2 Comparison of optical imaging and spectroscopy systems

Optical System	Resolution/size sensitivity	Penetration depth	Sources of contrast: information	Advantages	Limitations	Cost
Fluorescence spectroscopy	Probe and λ dependent (100s μm typical)	Probe and λ dependent (100s μm to few mm typical)	• Endogenous cellular fluorophores: cell biochemical, metabolic, differentiation status • Structural proteins: bulk matrix deposition, integrity, organization, remodeling • Exogenous labels: protein expression, cell lineage • Scaffolds, biomaterials: biomaterial status	• Simple implementation • Quantitative information on composition • Can be molecularly specific • Portable	• Low spatial resolution • Broad featureless spectra	Low
Elastic light scattering spectroscopy	Probe dependent (100s μm typical)	Probe and λ dependent (100s μm to few mm typical)	• Cellular organelles and membranes: cell structure, organelle packing • Collagen fibers: density/remodeling • Biomaterials, scaffolds: scaffold integrity	• Simple to implement • Portable	• Low spatial resolution • Not imaging based • Not molecularly specific	Low

Table 0-3 Physical comparison of optical imaging and spectroscopic techniques

Raman spectroscopy	Probe and system dependent (few μm to 100 μm)	Probe and λ dependent	Molecular bonds: biochemical composition, molecular structure	Molecular specificity	• Weak signal • Carefully designed fibers and/or detector required	Moderate
Confocal microscopy	Lateral: 0.5 μm or larger Axial: 1 μm or larger Both NA and λ dependent	10s μm to 300 μm	(Reflectance) • Collagen fibers, biomaterial scaffolds: detailed matrix morphology, organization, remodeling (Fluorescence) • Exogenous chromophores, FAD, collagen: cell presence, matrix morphology, blood flow/vascularization	• Depth-resolved imaging • Repeated monitoring possible	• Complex design • Limited depth resolution • Significant photobleaching • Cells usually visible with exogenous labels only	Moderate to high

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The comparative study presented demonstrate that the spectra and images are sensitive indicators of distribution of lipids, proteins, and carotenoids in breast tissue .the spectrum from spectroscopic methods such as Raman spectra contains a large number of peaks which characterized different band in molecules ,to identify the position of malignant cells surrounded by neoplastic normal cells in small size of tissue. The peak in tumor spectrum which the relation between Raman intensity and Raman shift (wavenumber) at this peak we convert wavenumber to wavelength, the tissue section thickness and small size was chosen to allow measurements determined by the choice of wavelength as the source laser, and use this wavelength in confocal microscopy which has raster scanning and construct the image which appear position of cells by different colours which is very useful in differentiate between malignant and noraml cells which lead to early diagnosis breast cancer tissue in early stage.

IV. Conclusion and Future Work

In summary, in this study we construct and build confocal microscopy and analyzed optical images which can classify tumors and normal breast tissues in small size of area due to scanning process and special characterization of pinhols in confocal microscope which focus on small size of area and produce a large number of pixels with high resolution, contrast and clear details in image.

Through the qualitative analysis of the Raman spectra and assignment of the relevant bands from the literature, it was possible to built spectral models and differentiates among normal breast, , duct carcinoma-in-situ,These differences were established through the comparative study between the spectral differences and the histo pathological diagnosis.

Furthermore, previous study able to establish the biochemical basis for each spectrum by relating the observed peaks to specific biomolecules that have special role in the carcinogenesis process.

This work is very useful for the precocious optical diagnosis of a broad range of breast pathologies and as far as we are concerned. We have found tha optical techniques (spectroscopic and microscopic) are complementary to each other by means spectroscopic technique are sensitive indicators of the distribution of main constituents of breast tissue structure such as lipids, proteins, fatty acids, carotenoids and water. But microscopic techniques are sensitive to determination the site of different cells in breast tissue be analyzing images which have high resolution. The confocal images show evidently that the noncancerous breast tissue contains a significant contribution from triglycerides originating from the adipose tissue filling the spaces around the lobules and ducts, and fatty acids that make up the cell membrane and nuclear membrane. The cancerous tissue is dominated by the protein component. The water amount confined in the cancerous tissue is markedly higher than in the noncancerous tissue.

A comparable spectral and spatial resolutions and measurement on the same samples, show the fundamental differences between the two complementary techniques. The coupling of an infrared spectrometer to an optical microscope offers the unique opportunity for studying biological samples with a spatial resolution limited by the near diffraction limit of infrared light applications of infrared microspectroscopic analysis.

For example Raman spectroscopy is an optical technique for measuring the vibrational energies in molecules. With Raman spectroscopy it is possible to identify which molecules are present in a sample. Raman spectroscopy is just one of many optical spectroscopy techniques for studying samples. The major potential of Raman spectroscopy compared to other methods is the possibility of easily identifying many different compounds in the same sample. If you combine Raman spectroscopy with imaging techniques you get Raman imaging. With Raman imaging it is possible to obtain information about the spatial distribution of molecular species within a sample. Due to the low cross section for Raman scattering, the Raman signals are extremely weak. This weakness results in that other scattering processes, such as fluorescence, may severely affect the possibility of performing Raman spectroscopy. To conduct Raman spectroscopy you therefore have to use methods to reduce fluorescence. One method is to use near-infra red excitation light, which has energy not high enough to cause fluorescence for most molecules.

Application of confocal microscopy studies in biomedical field is the ability to provide information on the identity, size, stereo-structure, time-change, substance diffusion, and concentration of fluorescent-labeled substances. Intracellular or membrane-bound fluorescent dyes as well as voltage- and calcium-dependent indicators are all used to investigate the functions and activities of cells.For cellular imaging, confocal optics provide a significant improvement in spatial resolution and data quantity. Confocal microscopy is being applied in neurobiology for detecting microstructures and activities within neurons. The technique is also used in clinics for disease diagnoses, in tracing pathological changes, and in studies of angiogenesis under several conditions. Furthermore, researchers in genetics use confocal microscopy to trace the expression of genetically encoded fluorescent proteins. The field of live-cell imaging has also greatly benefited from applications of confocal microscopy. Future developments in this field will be of great interest and benefit to both biotechnological and medical research.

The two techniques are different but complementary to each other for early diagnosis. The combination of an optical microscope, an optical spectrometer leads to the development of a new and powerful analytical characterization technology, denoted as infrared micro spectroscopy imaging, which can be used for the identification of normal and pathological states of tissues. This fascinating technology allows the generation of a huge amount of valuable data within a few seconds.

The first area for future work is to integrate the piezostage z-scan capabilities. One of the biggest advantages of confocal microscopy is the ability to image on well- defined planes. this allows images from multiple, parallelplanes to be reconstructed to produce a three –dimensional image .By using the z-scan capability of the piezostage,this method can be fully automated ,Furthermore ,this functionality is the already built into the mathwork-matlab used with the original image acquisition system.

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The biggest opportunity for further work involves improvement of math work-Matlab user interface. Graphical User Interface (GUI) is made in math work matlab program which is a powerful tool , The original confocal system had a very well developed user interface which is very familier to many of the systems most common operators., the new scanning system was designed to operate off of the same inputs as are provided through this well developed interface. Aditionally ,the system can be configured with minimal effort to output the same data as the confocal scanning does in the program used with the original system By performing the necessary modifications to matlab code to control the scanning system .it will be it simple and effective.

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