

To the Graduate Council:

I am submitting herewith a thesis written by Vijay Nandakumar entitled “Real-Time Monitoring and Quantification of Drug Induced Changes in Endothelial Cytoskeleton Filaments Using a Cellular Impedance Biosensor.” I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Engineering Science.

Anthony English
Major Professor

We have read this thesis
and recommended its acceptance:

Mehran Kasra

Mohamed Mahfouz

Accepted for the Council:

Anne Mayhew
Vice Chancellor and
Dean of Graduate Studies

(Original signatures are on file with official student records)

**REAL-TIME MONITORING AND QUANTIFICATION OF DRUG INDUCED
CHANGES IN ENDOTHELIAL CYTOSKELETON FILAMENTS USING A
CELLULAR IMPEDANCE BIOSENSOR**

A Thesis
Presented for the
Master of Science Degree
The University of Tennessee, Knoxville

Vijay Nandakumar
August 2005

DEDICATION

I primarily dedicate this thesis to my mother, father and my fiancée, without all of whom I would have never reached this stage. These are the people who consistently propelled me in life and made me realize my potential. Also I would like to dedicate this work to all my friends, both here in Knoxville and back in my home India for providing me support and healthy companionship.

ACKNOWLEDGEMENTS

I would like to extend my sincere appreciation and thanks to everyone who helped me complete this Master of Science degree. My first words of gratitude are definitely reserved for Dr. Anthony English , who guided me with his innate brilliance and patience that opened me the gates to hardcore research and helped me complete my Master's work in a organized and timely manner. He was instrumental in making me realize as to what it takes to achieve success at the highest level and backed me up to try out novel ideas. I would also like to thank Dr.Mohammad Mahfouz, who with his expertise and accessibility, was always there for any queries in the image and signal processing areas. I extend my sincere thanks for Dr. Mehran Kasra, who always has a pleasant smile and bright ideas, for serving in my committee and guiding me towards my goal.

I would also like to extend my thanks to Dr. John Dunlap, who helped me a lot in the confocal imaging sessions and Dr. Uday Kumaraguru, who helped me in making the buffers and formulate protocols for staining studies. I also extend my appreciation and thanks for all the faculty members who handled my courses and the administrative staff in my department and the Graduate school for making my experience here a rewarding one.

Finally, I extend a special thanks to my father, who is my source of inspiration and a role model who made me believe that I can achieve in life.

ABSTRACT

The analysis of electrical signals originating from biological cells and tissues have yielded a large amount of useful information over the past years. Technologies have been developed, wherein we can monitor the health of the biological material of interest using these electrical signals. Instruments for the study of living cells have historically been of significant importance for such things as basic neuroscience, cell biology, pharmaceutical screening, environmental monitoring, and toxin detection. One of the practical realizations of these methods was successfully implemented by Giaver and Keese with their Electric Cell-Substrate Impedance Sensing (ECIS) system. The purpose of this Masters Thesis is to showcase the use of a Cellular Impedance Biosensor setup based on the ECIS system in real-time monitoring of endothelial barrier function and quantify the dynamic changes of the cytoskeleton filaments induced by different drugs. The filaments of the endothelial cell cytoskeleton play a critical role in cellular micro motion and the inflammatory response. The fact that the cellular cytoskeleton is the essential force behind all the motile activities of the cell is used in drug discovery methods to design ways so as to kill tumor cells and developing cell-based therapies for different cardiovascular pathologies. Cytochalasin D was chosen to study the actin filament response as the drug specifically inhibits the polymerization of actin filaments, which play a crucial role in the cellular mechanical strength and micromotion. Nocodazole was

chosen to study microtubules, which play a pivotal part in the cellular mitotic activities and locomotion. These drugs were chosen as they have extreme effects on the cytoskeleton and would be ideal to showcase the use of the biosensor in tracking the intracellular dynamics. The biosensor system provides a simple interface for monitoring the electrical activity and impedance characteristics of populations of cultured cells over extended periods. The cell - sensor interface is created as cells attach to the gold electrode surface pre-coated with fibronectin. Using these impedance measurement techniques based on a simple cellular geometric model, we have been able to successfully monitor cellular adhesion, motility, proliferation and changes in the cellular cytoskeleton induced by different drugs as mentioned above. The kinetic response of the cellular cytoskeleton to different doses of these drugs is translated as changes in the impedance measured by the biosensor setup. The data obtained from the setup were quantified by correlating them with the images obtained by confocal microscopy. A wavelet transformation algorithm is applied to the acquired data in an attempt to capture the fluctuations and compare the cellular behavior before and after the addition of drugs.

Study of the disruption of these filaments by toxins and pharmacological agents holds lot of promise to provide a model for studying their role in endothelial cell biomechanics and the pathology of cardiovascular, pulmonary, and renal disease. This thesis presents a cellular impedance biosensor setup that can carry out this study in real time with high sensitivity and reproducibility. Using these impedance measurement techniques, we have

been able to successfully monitor cellular adhesion, motility, proliferation of different type of cells and their response to external stimuli.

TABLE OF CONTENTS:

Part I: Introduction

I. Introduction.....	2
A. The Cell and its Cytoskeleton.....	2
B. The Electrical nature of the Cell	3
C. Electrical Analysis of the Cell.....	6
D. The Electrode Cell-Substrate Impedance Sensing (ECIS) system.....	8
II. Motivation and Problem Statement.....	10
III. Thesis Outline.....	11

Part II: The Cellular Biosensor Setup and Impedance Measurements

ABSTRACT.....	14
I. Introduction.....	15
II. Methods and Materials.....	16
A. Endothelial Cell Isolation and Preparation.....	16
B. An Electric Model of the Cell.....	17
C. The Electrode Arrays.....	21
D. Electrode Inoculation.....	22
E. Lock-in Amplifier.....	22
F. Data Acquisition System.....	24
G. Impedance Biosensor Setup.....	25
III. Results and Discussions.....	27
IV. Conclusions.....	31

Part III: Real-Time Monitoring and Quantification of the effect of Cytochalasin D on PPAEC using the Cellular Impedance Biosensor

ABSTRACT.....	33
I. Introduction.....	35
II. Methods and Materials.....	37
A. Cell Culture and Actin Cytotoxicity Assay.....	37
B. Immunofluorescence of the Actin Cytoskeleton.....	40
C. Laser Confocal Microscopy.....	41
D. Wavelet Transforms.....	44
III. Results and Discussions.....	46
IV. Conclusions.....	51

Part IV: Real-Time Monitoring of Microtubule Disruption using the Biosensor Setup

ABSTRACT	53
I. Introduction.....	54
II. Methods and Materials.....	56
A. Tubulin Cytotoxicity Assay.....	56

B. Immunofluorescence of the Microtubules.....	57
III. Results and Discussions.....	58
IV. Conclusions.....	60
Part V: Future Considerations and Conclusions	
I. Introduction.....	62
II Future Considerations.....	63
A. Real-time Monitoring of Intermediate Filament Dynamics.....	63
B. Monitoring Cellular Behavior on Artificial Surfaces.....	64
III. Part Conclusions.....	67
IV. Thesis Conclusions.....	68
References.....	70
Appendices.....	75
Appendix I: Figures.....	76
Appendix II Programs and Tables.....	115
Vita.....	124

List of Figures

Figure A.1.1: Cells modeled as round disk shaped objects and the corresponding resistances R_b , α and the capacitance across the cell membrane are represented. The current flow through the cell-electrode interface depends on these three parameters.....	77
Figure A.1.2: The path of current flow through the endothelial cell-substratum boundary and the equivalent electrical circuit along with the impedances, current density and voltage drops at different junctions.....	78
Figure A.1.3: The Base electrode which forms the cardinal part of the biosensor setup along with the series of five gold microelectrodes housed inside the plastic wells.....	79
Figure A.1.4: The structure and surface properties of the microelectrode in conjunction with the counter electrode is represented.....	80
Figure A.1.5: Micrograph of the gold measuring electrode with no cells on it.....	81
Figure A.1.6: Figure A.1.6: Micrograph of the cultured endothelial cells and the Cytoskeleton structure	82
Figure A.1.7: A typical block diagram of a Lock-in Amplifier is represented. The lock-in amplifier is used here to stimulate the cells using a non-invasive current of 1 μ A and also phase-sensitively detect the cellular response	84
Figure A.1.8: The National Instruments DAQ system is shown here. We used the SCXI 1000 series which collected the data from the biosensor through the lock-in amplifier according to instructions set by the Virtual Instrumentation program coded in LabVIEW.....	85
Figure A.1.9: Micrograph showing the endothelial cell adhesion on top of the measuring electrode. It has to be noted that this formation of a monolayer on top of the microelectrode is the underlining principle of this whole biosensor setup and provides a non-invasive approach to monitor cells <i>in vitro</i>	86
Figure A.1.10: The Cellular Impedance Biosensor Setup	87

Figure A.1.11: A digital picture representing the whole instrumentation setup in the lab that we used for our study.....	89
Figure A.1.12: The graph shows the naked and cell covered resistance and reactance as a function of frequency for the initial scan.....	90
Figure A.1.13: The graph shows the naked and cell covered resistance and capacitance as a function of frequency after 25 scans. The difference between the cell covered and naked electrode scans can be easily seen between the 1-10 KHz range.....	91
Figure A.1.14: The graph shows the naked and cell covered resistance and capacitance as a function of frequency after 50 scans. The difference between the cell covered and naked electrode scans is more pronounced between the 1-10 KHz range.....	92
Figure A.1.15: A series of normalized impedances as a function of frequency elucidating the effect of formation of the monolayer on top of the electrode. It can be seen that after a complete attach the impedance measured can be as high as 6-8 times the naked electrode impedance.....	93
Figure A.1.16: A time series of the naked and cell covered electrode scans. The fluctuations in the acquired data should be noted here. These fluctuations correspond to the cellular micromotion on top of the microelectrode after formation of a confluent layer.....	94
Figure A.1.17: The figure represents the layout of a typical confocal microscope. The special feature of the confocal microscope is its ability in extracting optical slices of samples that is not possible with a traditional microscope.....	95
Figure A.1.18: The principle behind the confocal microscope in obtaining 3-D representation of the sample by collecting slices across different sections of the sample.....	96
Figure A.1.19: The figure shows a screenshot of the LabVIEW Virtual Instrument code for the wavelet transformation in order to capture the cellular fluctuations	97
Figure A.1.20: The figure represents a series of data obtained after addition of Cytochalasin D in concentrations ranging from 30uM to .003uM.	98
Figure A1.1.21: As we can see from the results the data we obtained was reflective of cellular behavior but with a irregular pattern between 3uM and .3uM	99
Figure A.1.22: The figure represents the data series obtained at a frequency of 5.62 KHz and treating the cells with concentrations of cytochalasin logarithmically spaced between 3.16 μ M and 0.316 μ M.....	100

Figure A.1.23: This figure is represented so as to give the user a better understanding of the biosensor in effectively following the intracellular mechanics for different concentrations of Cytochalasin D.....	101
Figure A.1.24: The Normalized data for the actin cytotoxicity assay zoomed to the point just after the addition of the drug to given an idea about the reaction kinetics for the different concentrations.....	102
Figure A.1.25: The image is that of an actin stained endothelial cell obtained using the laser confocal microscopy technique. The individual filaments being clearly visible reflects the good staining protocol followed.....	103
Figure A.1.26: A series of confocal images that show the actin disruption for each of the drug concentration used. The filament density can be easily correlated with the drug concentration and this in turn can be correlated with the acquired biosensor data. The confocal microscopy technique is used here as a means of quantifying the impedance data with respect to the drug concentration.....	104
Figure A.1.27: A graph of the actin filament density plotted against the drug concentration. The filament density is obtained by applying standard image processing techniques by programming in Matlab.....	105
Figure A.1.28 (top to bottom): The wavelet transformed representation of the acquired data for concentrations of 3.16 μM , 1.78 μM , 0.562 μM and the attach scan. The various area markings reveal the cellular response as explained in the discussion.....	106
Figure A.1.29: The figure represents the initial time series data obtained for the nocodazole cytotoxicity assay at concentrations of 100 μM , 56.2 μM , and 10 μM	107
Figure A.1.30: The series of graphs represent the data acquired after treating the cells at modified concentrations ranging between 3.16 μM -10 μM	108
Figure A.1.31: The collective data representing the response of PPAEC to Nocodazole at concentrations mentioned above. This is to give a better understanding of the reaction kinetics at different concentrations of nocodazole.....	109
Figure A.1.32: The figures (top to bottom) represent the wavelet transformed data obtained for concentrations of 10 μM , 5.62 μM and the attach scan respectively. The interpretation is similar to that of the wavelet transform for Cytochalasin D data.	110

Figure A.1.33: The figure represents the difference in response of the cellular cytoskeleton to different drugs. The varying response detected by the sensor for the same concentration of different drugs is represented here.....	111
Figure A.1.34: A series of confocal images obtained while attempting to visualize the microtubules. It can be seen that the tubules are not clearly visible as the staining protocol is still under optimization.....	112
Figure A.1.35: The figure represents the flow process in the design of a microchannel. This is the proposed process for the PDMS based impedance biosensor.....	113
Figure A.1.36: The figure represents the perspective views of the proposed PDMS based cellular impedance biosensor. This arrangement can be used to monitor the cellular activity on artificial surfaces such as hydrogels.....	114

Part I
INTRODUCTION

I. Introduction

A. The Cell and its Cytoskeleton

The cell is one of the most basic units of life. There are millions of different types of cells. There are cells that are organisms onto themselves, such as microscopic amoeba and bacteria cells. And there are cells that only function when part of a larger organism, such as the cells that make up our body. There are more than 200 different types of cells that are responsible for various functions of our body. The cell has been the object of research for many investigators as it provides useful information on the pathology of diseases, infections and other health related phenomenon.

The Cytoskeleton forms the most important part of the cell. It is a dynamic internal framework/scaffold coordinating cytoplasm and its components. It is primarily composed of three filamentous elements: microtubules, microfilaments, & intermediate filaments which contribute to cell shape and act in cell division, muscle movement and locomotion. Actin Filaments are formed by the polymerization of the protein actin (the most abundant protein in the cell) into long, thin fibers. These are about 8 nm in diameter and, being the thinnest of the cytoskeletal filaments, are also called microfilaments. Since they form a band beneath the plasma membrane, they provide mechanical strength to the cell and link the transmembrane proteins to the cytoplasmic proteins. Microtubules are a set of another important filament constituting the cytoskeleton and essentially act as conveyer belts inside the cell. They are composed of subunits of the protein tubulin--these subunits are termed alpha and beta. Microtubules act as a scaffold to determine cell shape, and provide a set of "tracks" for cell organelles and vesicles to move on. Microtubules also

form the spindle fibers for separating chromosomes during mitosis. When arranged in geometric patterns inside flagella and cilia, they are used for locomotion. Intermediate filaments are about 10 nm (and thus are "intermediate" in size between actin filaments (8 nm) and microtubules (25 nm)) diameter and provide tensile strength for the cell by acting as a supporting framework.

The cytoskeleton thus is essentially what the cell uses to move. The study of these intracellular filaments and their mechanics along with the drugs used to block their movement has been of great use in the treatment of cancer cells [1]. Understanding the dynamics of these filaments and the cytoskeleton in general has a great potential use in the development of drugs against various pathological conditions like cancer, atherosclerosis and other cardiac and respiratory disorders.

B. The Electrical nature of the Cell

The small size has made the study of cells difficult until the invention of light microscope. However, mammalian cells are not only tiny but also translucent and colorless. Techniques for understanding the finer structure of cells weren't developed until the latter part of the 19th century, when stains that can provide sufficient contrast became available. The study of cellular function and response has been used for decades to better understand the workings of the body. As technology has advanced, the capabilities and sensitivity of cellular studies have greatly improved. One such technology is studying the cell properties by stimulating it with an electrical signal and

analyzing its response. This unique technique of measuring the cellular electrical response has become a whole new way of looking at cells.

Much of the intricate and complex behavior of organisms can be attributed to the electrical activity of the cell. Various processes like sensory perception, cardiac function, muscle control, thought, etc. are all partially controlled and regulated by the electrical activity and response of different cells in the body. Structurally, cells are composed of a lipid bilayer membrane enclosing an intracellular ionic solution. This membrane contains numerous proteins, receptors, ionic channels, and ionic pumps which are responsible for maintaining the ionic concentrations within the cell and the intracellular potential relative to the extracellular potential [2].

Luigi Galvani of Italy is often credited with the establishment of electrophysiology of nerve tissue [3]. Galvani published the results of his eleven years of experimentation in 1791. From Italy his work spread to Germany in the research of Emile du Bois-Reymond and Hermann von Helmholtz. Most of du Bois-Reymond's work concerned nerve excitation, and he is credited with measuring what he called "negative Schwankung" or negative variation. This measurand was later termed action current or action potential. In 1850, a contemporary of du Bois-Reymond discovered a way to measure the speed of propagation of a nerve signal. Hermann von Helmholtz devised an innovative apparatus for his experiment. Time elapsed from the application of the electric stimulus until the physical contraction of the muscle was measured. The actual movement of the muscle opened the electric circuit and thereby terminated the application of the electric stimulus. This work was continued by two students of du Bois-Reymond, Julius Bernstein and Ludimar Hermann. Hermann proposed a theory that the signal could be propagated by a

current flowing from an exciting section of the nerve to a section of the nerve that was initially at rest, Hermann local circuit theory. Bernstein was the first to measure the time course of the action current. Bernstein also applied the idea of a selectively permeable membrane and the diffusion of ions to account for the difference in potential between the inside of cell and the outside.

The major advances in electrophysiology in the twentieth century began in 1934. This is when Alan Hodgkin began his research into the physiology of nervous tissue. Hodgkin's first experiment resulted in confirming Hermann's local circuit theory. Some time later, Cole and Curtis and Hodgkin and Huxley all recorded the transmembrane potential of the giant squid axon. Using this same type of axon in 1949, Hodgkin and Katz explained that the action potential of the cell membrane was due to an increase in membrane permeability to sodium ions. At rest the membrane was permeable only to potassium ions; however, in an excited state, the influx of sodium dominated the action potential. In 1949, the invention of the voltage clamp by Cole and Marmont gave researchers a new tool for the study of electrophysiology. This new tool was used in 1952 by Hodgkin, Huxley, and Katz to further interpret the biological events behind the action potential. However their research did not answer all the questions concerning cellular electrophysiology. Researchers were still unsure what controlled ion flow through the membrane, a pore-like opening in the membrane or some transporter molecule. The idea of an ion channel was introduced by Erwin Neher and Bert Sakmann in 1976 based on their work with patch clamps. This discovery helped to further the scientific knowledge of electrophysiology.

C. Electrical Analysis of the Cell

Right throughout the last decade, elaborate experimental methods to isolate and culture mammalian cells in vitro have been developed and constantly improved so that cell cultures from almost any tissue of the human body are available today[13]. This has been largely motivated by the perspective to study one particular cell type apart from the complexity of an entire organism under well defined conditions in a laboratory environment. However, for a maximum of scientific information it is not sufficient to have the cell cultures available, it is equally important to develop sensitive laboratory techniques to either monitor the behavior of living cells non-invasively by using sensors or to manipulate them using actuators.

With the development of planar microelectrode arrays and advent in micro fabricating technologies, measurement of electrical activity of the cell has become a common method for inference of various cellular processes [2]. Of all the methods involving the analysis of electrical signals from these biological specimens, impedance measurement technique is the most widely used to monitor cellular activity. Impedance is the frequency-dependent opposition of a conductor to the flow of an alternating electric current. A measure of impedance (Z) is composed of the sum of two vectors, resistance (R) and reactance (X_c), measured at a particular frequency and is described mathematically by the equation $Z^2 = R^2 + X_c^2$. Resistance is the opposition of a conductor to the alternating current, and as the electric current travels through the body, resistance is basically the same as in nonbiological conductors [3]. Reactance is produced by the additional opposition to the current from the capacitant (storage)

effects of cell membranes, tissue interfaces, and structural features [4]. Early studies of bioelectrical impedance focused on the meaning of impedance measures in relation to the water and electrolyte content of the body and to physiological variables such as thyroid function, basal metabolic rate, estrogenic activity, and blood flow in human and animal tissues [5]. These explorations developed into some of the present-day areas of impedance cardiography, pulmonary impedance, brain impedance, and impedance imaging. The use of bioelectrical impedance to estimate body composition developed from more recent exploratory works in the areas of single-frequency and multiple-frequency impedance. An alternating current is used for bioelectrical impedance analysis because it penetrates the body at low levels of voltage and amperage. In a complex electrical structure such as the human body, the part of the fluid volume or TBW measured by bioelectrical impedance is also a function of the current frequency. At low frequencies of less than about 5 kHz, the bioelectrical current travels primarily through extracellular fluids, but as the frequency increases, the current starts to penetrate body tissues, creating reactance. At high frequencies (above 100 kHz), the current is assumed to penetrate all conductive body tissues or all of the total body water in the conductor and supposedly overcomes the capacitive properties of the body, reducing reactance to zero. One of the earliest devices was manufactured by Hanna and Johnson (1968) who produced an array of 20 or 30 electrodes on a plastic sheet using photo etching techniques. Their device however was intended for surface potential recording due to the size of the electrodes. Although designed for recording from cardiac cells, one of the original microelectrode devices manufactured was by Thomas *et al.*[6]. The basic fabrication principles of this method are still used in more modern

designs. This microelectrode array was etched into thin metal films deposited on glass cover slips. Thin-film nickel layers were vacuum deposited onto a glass coverslip and were insulated with a thin layer of insulating polymer. The coverslip was then exposed to light through a photographic negative image of the desired electrode pattern. The polymer was removed and the metal remaining uncovered was removed with acid. The nickel was then gold-plated, re-insulated, and the electrodes exposed by repeated light exposure with a second negative image of the desired pattern. Electrode impedance was improved by coating the electrode tips with platinum black. Their system contained 30 microelectrodes arranged in two rows 50 mm apart; the distance between electrodes in rows was 100 mm. Using this system Thomas and colleagues (1972) were able to record potentials from cultured chick cardiac cells. Successful implementation of the microelectrode system by Thomas et al. inspired many investigators to extend its use to monitor the electrical activity of many organisms like bacteria, algae [9], neurons [8] and also various organs in our body [10-12]. With the rapid development of fabrication techniques and advent of visualization methods, monitoring the health of cells and tissues and following their dynamics has provided a plethora of information, thus solving many mysteries which have puzzled investigators over the years. Yet, it is predicted that there is a long way to go before we can completely understand the complexity of cellular function and activity.

D. The Electrode Cell-Substrate Impedance Sensing (ECIS) system

The Electric Cell-substrate Impedance Sensing system is a novel biosensor setup designed for real time monitoring of cell behavior including attachment, motility and

cytotoxicity in a continuous fashion. It was developed by Drs. Ivar Giaever and Charles R. Keese [14-15] in 1984 and commercialized in the early 90`s. The novelty in the ECIS setup was stimulating the cell by a weak electric signal and analyzing its response which was completely different from the traditional method of recording and analyzing the inherent electrical property of the cell. This method thus provided a new dimension in cell research which allowed the continuous tracking of cellular adhesion, growth and micromotion and even the cellular response to toxic substances and pharmacological agents [16-18].

This technology subjects cells growing on gold microelectrodes to small ac electric fields. Since the cellular bodies behave essentially like insulating particles, a very weak and non-invasive current ($< 1 \mu\text{A}$), is used to perform the measurement. Upon the attachment and spreading of cells, this current has to flow around the cell which increases the measured electrical resistance of the electrodes [13]. An intriguing feature is the fluctuation in the measured impedance, which is always associated with living cells and persists even if the cell layer becomes fully confluent. This behavior is attributed to vertical motions or micromotion of the cells, an indication of cell viability and morphology change. The resistance read out is dependent on electrode coverage and – in case of confluent cell layers – on the three-dimensional shape of the cells. Since the electrodes are very close to the cell bodies, this electrical measurement is very sensitive and capable of resolving changes in cell shape much smaller than the resolution of an optical microscope.

The surface of the gold electrodes is usually precoated with proteins. Initial intact extracellular matrix binding to cells is often mediated through adhesion receptors in the integrin family with fibronectin as the commonest coating protein. This allows the PPAEC adhere to the surface of the electrodes and form a monolayer similar to the layer over the blood vessels and arteries *in vivo*.

II. Motivation and Problem Statement

It was in the early 1990s that Applied Biophysics was formed by Drs. Ivar Giaever and Charles R. Keese to commercialize the ECIS system and since then many investigators have used the sensor to study the different intricate properties of the cell. The ECIS system has been used for a wide variety of applications like determining the cell culture confluence [17,19], monitoring the growth of cells under different conditions [20-22], monitoring the behavior of cancerous cells [23,25,26], cytotoxicity assessment of cells [18,21] and monitoring the wound healing of cells [27,28] to name a few. Since its inception, the ECIS system has been studied in depth by investigators [30, 31] to quantify the results further. The system has thus been actively pursued by investigators to understand the different complex properties and response of the cells.

The importance of the cytoskeleton has been reviewed by many investigators over the years [32-36, 38]. Active investigations of the cytoskeleton and its role in cellular adhesion, mobility, proliferation and the change in the cytoskeleton dynamics under various pathological conditions has been studied by researchers. Thus a study of cytoskeleton dynamics under different conditions and its response to various drugs and pharmacological agents holds lot of promise to provide useful data about disease

mechanisms. Many studies have attempted to use a combination of different techniques to view and analyze the cytoskeleton [37, 39] and researchers, of late have shown interest in following the dynamics of the cytoskeleton filaments during naturally occurring cellular processes [40-43], but, only a limited number of studies have been carried out on the cellular response and change in the cytoskeleton dynamics in response to external sources and its effect on the cell [44- 46].

Thus, the purpose of this master's thesis is to devise a protocol to continuously track the reorganization of the cytoskeleton in response to external signals and agents through electrical measurements and other supporting techniques. The work thus aims to provide a model for the response of the cytoskeleton proteins to different toxins and drugs *in vivo* which is pivotal in the motile activities of the cell, which in turn determines the very function of various parts of the body.

III. Thesis Outline

This Master's thesis work aims to track the continuous changes in the filaments of the cellular cytoskeleton and its effect on the cell through a Cellular impedance biosensor setup and optical techniques. Specific drugs are chosen to inhibit or act against specific filaments of the cytoskeleton to analyze how it affects the behavior of the cell. The dynamics of the cytoskeleton response are tracked for each filament specific drug using the real time measurements from the biosensor and data from the biosensor is correlated with the optical data as will be discussed in the reminder of the thesis. The entire thesis is structured in a way that each part with the exception of introduction and conclusions follow the format of a professional publication.

The thesis is structured with an Abstract to start, which is followed by a general introduction. Part 2 deals with the definition of the biosensor system and the modules followed to acquire and analyze data from it. The measurements are made under normal conditions to give an overview of the instrumentation. Part 3 discusses the real-time monitoring of the actin dynamics using the sensor and its correlation to the imaging data. A wavelet analysis program coded in LabVIEW is applied to the acquired data to identify the fluctuations. Detailed descriptions are given about the experimental setup, sample preparation and analysis techniques. The microtubule dynamics are investigated in real-time in part 4. The response of the cell to different drugs is measured electrically and correlated with the images. This section is structured in a similar manner to that of part 3. Part 5 deals with the future scope, applications of this work and general conclusions.

PART II:

The Cellular Biosensor Setup and Impedance Measurements

ABSTRACT

This section deals with introduction to the theory and methods behind the cellular impedance biosensor setup based on the ECIS system and carries an investigation of the impedance measurements. Electric cell-substrate impedance sensing (ECIS), has become increasingly used to study and quantify endothelial cell physiology in real time [13-17]. The cellular impedance biosensor is devised based on the ECIS principle. The method is based on stimulating the cells with a non-invasive current over a range of frequencies and measuring changes in AC impedance of small gold-film electrodes deposited on a culture dish that is used as growth substrate for the cells. Based on a simple cellular geometric model, frequency dependent impedance measurements obtained from this sensor are being used to estimate the cell-cell and cell-matrix impedance components of endothelial cell barrier function and thus follow the various motile activities of the cell like adhesion and proliferation, which are translated as changes in the measured impedance and fluctuations. On analysis, it is determined that the cellular activity is effectively followed at a frequency range of 4-10 KHz. The cells adhere to the electrodes in a manner which in the basic sense similar to the adhesion mechanism in vivo and the analysis of this data provides a solid foundation to carry out further investigations.

I. Introduction

The endothelium is located at the interface between the blood and the vessel wall. The cells are in close contact and form a slick layer that prevents blood cell interaction with the vessel wall as blood moves through the vessel lumen. The endothelium consists of simple squamous epithelium that lines the capillary of all blood vessels. It plays a critical role in the mechanics of blood flow, the regulation of coagulation, leukocyte adhesion, and vascular smooth muscle cell growth, and also serves as a barrier to the transvascular diffusion of liquids and solutes. For years the endothelium was thought of as an inert single layer of cells that passively allowing the passage of water and other small molecules across the vessel wall. However, this dynamic tissue performs many other active functions, such as the secretion and modification of blood vessel regulating substances and the contraction and relaxation of vascular smooth muscle.

Transendothelial cell monolayer impedance is a complicated and sensitive function of cell-cell adhesion, cell-matrix adhesion and the cellular membrane properties. As a result, endothelial cell monolayer impedance measurements have the potential to provide valuable insight into a number of cellular processes. The adhesion of endothelial cells to each other and the underlying matrix, for example, plays a critical role in the optimal development of tissue engineered vascular constructs and the etiology of many cardiovascular, pulmonary and renal diseases.

Electrical impedance measurements coupled with biotransport and cellular biochemical methods have emerged as a powerful tool for investigating endothelial cell barrier

function in terms of receptor-ligand interactions and signal transduction pathways. The quantification of endothelial barrier function and its interaction with artificial substrates have been actively pursued by investigators which resulted in the design of a novel cellular impedance biosensor referred to as the Electric Cell-substrate Impedance Sensing (ECIS), designed and developed by Drs. Ivar Giaever and Charles R. Keese in 1984. The ECIS has then been widely used to study the endothelial cell physiology in real time and cited in a myriad of scientific journals and publications.

A simple geometrical model of the endothelial cell is presented for a better understanding of the cellular response to electric stimuli. The impedance measurements are carried out over a wide range of frequencies and the cellular response is monitored. The impedance changes as a function of the cellular behavior and interaction with the electrode. The remainder of this part discusses the various procedures and protocols to setup the system and carry out the basic impedance measurements. The potential of this biosensor setup is realistically wide as the Cellular impedance setup provides a solid foundation for carrying out further investigations on the intricate intracellular architecture.

II. Methods and Materials

A. Endothelial Cell Isolation and Preparation

Pulmonary endothelial cells were isolated from porcine pulmonary arteries obtained from a local abattoir. The arteries were resected and the ends were clamped using a hemostat. It was treated in 70% ethanol and then rinsed thoroughly with M199 (Gibco BRL). The arteries were then transferred back to the laboratory in M199 containing penicillin (100

U/mL) and streptomycin (100 μ g/mL) (Gibco BRL). Each artery was then dissected open with sterile scissors and the layer of endothelial cells were carefully scrapped off from the luminal surface using a sterile scalpel. The scalpel was then gently tapped on the surface of a 35 mm Petri dish which enables the cells to be transferred on to the dish which contained 2 mL of 50:50 conditioned M199. All cells were cultured using M199 containing penicillin and streptomycin, supplemented with L-glutamine (Gibco BRL), BME amino acids (Sigma), BME vitamins (Sigma) and fetal bovine serum (Hyclone). The media in the Petri dish was replaced with fresh reconditioned M199 every four hours. The culture was thus maintained for about a week after which the cells reached confluence. The endothelial cells were then passaged into a 60 mm tissue culture dish when near confluence. After about a week the cells reach confluence and they were now transferred on to a 100 mm tissue culture dish. The endothelial cells were then regularly passaged once a week at a ratio of 1:4 or 1:5. Porcine pulmonary artery endothelial cells between passages four and eight were used for this study and care was taken to not extend the cell lines beyond passage 12.

B. An Electric Model of the Cell

The impedance due to the cell layer can be mathematically modeled, where the data obtained can be used to calculate the morphological parameters of the cell such as the monolayer barrier function, the cell membrane capacitance and the ventral spacing of the cell-substratum interface [17]. In this model, the cells are represented as round disk shaped objects as shown in the figure A.1.1. The cell membrane is considered as an insulating surface and the cell is filled with the conducting electrolyte. The cells are

assumed to be hovering above the electrodes which act as the substratum. The conductance path of the endothelial cell can be seen from figure A.1.2. The impedance R_b represents the intercellular resistance path between adjacent cells. As adjacent cells pull apart, or the degree of cell-cell adhesion decreases, R_b decreases. The transcellular pathway is modeled as two cell membranes in series with a membrane capacitance of C_m and a parallel resistance of R_m , thus contributing to a total impedance of Z_m . An additional resistance that represents the resistive path underneath the cell depends on the media resistivity, ρ , and the subcellular separation distance, h . Increasing cell-matrix adhesion that decreases the subcellular space increases the subcellular resistance.

To determine the potential field equation underneath a cell, consider a subcellular current density, $\mathbf{K}$, and potential, V , that varies only in the $x^1 - x^2$ plane as shown in Fig.(). The current and potential at the cell-electrode interface is assumed to be constant in the x^3 direction. The electric field, $\mathbf{E}$, produces current densities at the electrode electrolyte interface and the subcellular interface, J_n and J_m , respectively, are directed in the x^3 direction. The governing field equations are

$$\mathbf{E}(x^1, x^2) = \frac{\rho}{h} \mathbf{K}(x^1, x^2) \Rightarrow -\nabla_{x^1 x^2} V(x^1, x^2) = \frac{\rho}{h} \mathbf{K}(x^1, x^2), \quad (1)$$

$$V_n - V(x^1, x^2) = Z_n \mathbf{J}_n(x^1, x^2), \quad (2)$$

$$V - V_m(x^1, x^2) = Z_m \mathbf{J}_m(x^1, x^2), \text{ and} \quad (3)$$

$$\nabla_{x^1 x^2} \cdot \mathbf{J}(x^1, x^2) = \mathbf{J}_n(x^1, x^2) - \mathbf{J}_m(x^1, x^2), \quad (4)$$

Where ρ is the solution resistivity, $Z_n(\nu)$ the specific impedance of the electrode-

electrolyte interface, and $Z_m(\nu)$ is the specific impedance of the cell membrane. These equations are equivalent to the partial differential equation [17].

$$-\nabla^2 V - \gamma^2 V + \beta = 0, \quad (5)$$

$$\text{Where, } \gamma^2 = \frac{\rho}{h} \left(\frac{1}{Z_n} + \frac{1}{Z_m} \right) \quad \text{and} \quad \beta = \frac{\rho}{h} \left(\frac{V_n}{Z_n} + \frac{V_m}{Z_m} \right). \quad (6)$$

By redefining the scalar potential function with an additive constant such that

$$V' = V - \frac{\beta}{\gamma^2}, \quad (7)$$

Eq. 5 becomes the Helmholtz equation

$$\nabla^2 V' + \gamma^2 V' = 0. \quad (8)$$

In cylindrical coordinates $x^1 = r$ and solutions to Eq. 8 are of the form

$$V = AI_0(\gamma r) + \frac{\beta}{\gamma^2}, \quad (9)$$

Where I_0 is the modified Bessel function of the first kind of order 0. Modified Bessel functions of the second kind are not included in the general solution since they go to infinity as $r \rightarrow 0$.

The total current flowing out of the electrode under a single cell, I_{ct} , of radius r_c can be found by integration, i.e.,

$$I_{ct} = \int_0^{r_c} \frac{2\pi r}{Z_n} (V_c - V) dr = -\frac{2\pi r_c^2 A}{Z_n r_c} I_1(\gamma r_c) + \frac{\pi r_c^2}{Z_n + Z_m} V. \quad (10)$$

The total current flowing through the cell membrane, I_{mt} , is

$$I_{mt} = \int_0^{r_c} \frac{2\pi r}{Z_m} (V - V_m) dr = \frac{2\pi r_c^2 A}{Z_m r_c} I_1(r_c) + \frac{\pi r_c^2}{Z_n + Z_m} V_n, \quad (11)$$

where $V_m = 0$.

The constant A is found from the boundary conditions, i.e.,

$$V(r_c) = (I_{ct} - I_{mt}) \frac{R_b}{\pi r_c^2} = -\frac{2}{r_c} A I_1(r_c) R_b \left\{ \frac{1}{Z_n} + \frac{1}{Z_m} \right\}. \quad (12)$$

Since

$$Z_c = \pi r_c^2 \frac{V_n}{I_{ct}} \rightarrow \frac{1}{Z_c} = \frac{1}{\pi r_c^2} \frac{I_{ct}}{V_n} \quad (13)$$

this gives us the closed form solution previously published by Giaever and Keese[]

$$\frac{1}{Z_c} = \frac{1}{Z_n} \left[\frac{Z_n}{Z_n + Z_m} + \frac{\frac{Z_m}{Z_n + Z_m}}{\frac{r_c}{2} \frac{I_0(r_c)}{I_1(r_c)} + R_b \left(\frac{1}{Z_n} + \frac{1}{Z_m} \right)} \right], \quad (14)$$

where $I_0(r_c)$ and $I_1(r_c)$ are modified Bessel functions of the first kind of zero and first order, respectively, and r_c is the radius of a single cell. The solution depends on R_b , the resistance between the cells for a unit area, and α , where

$$\alpha = r_c \sqrt{\frac{\rho}{h}}. \quad (15)$$

To calculate the specific resistance for cell-cell and cell-matrix adhesion, the current is assumed to flow radially from under the ventral surface of the cell and the electrode and then escape between cells. A minor amount of current goes directly through the cell

membrane by capacitive coupling or transcytoplasmic shunting. In this model, the total impedance across a cell-covered electrode is composed of the impedance between the ventral surface of the cell and the electrode (related to Z_c), the impedance between cells (indicated by R_b), the transcellular impedance (Z_m), and the impedance of the naked electrode Z_n . The specific impedances, Z_n and Z_m , are frequency dependent. Since Z_n and Z_c are measured and Z_m is the impedance of the two cell membranes in series, Z_c , R_b and Z_m , or C_m , are the only adjustable parameters in Eq. 14.

C. The Electrode Arrays

The electrodes are bought from Applied Biophysics (Troy, NY) and are embedded in as a series of five wells as shown in A.1.3. These disposable electrode arrays consist of gold film electrodes which are coated with an insulating film. The gold film is made sufficiently thin so that observation of the cells with a microscope is possible. Each well has a gold electrode at the base which is 250 μm in diameter and has a surface area for cell attachment and growth of 0.9 cm^2 . Current flows through this small electrode and a larger counter electrode. Usually, the counter electrode is made 10^2 - 10^3 folds larger than the measuring electrode so that the impedance of the measuring electrode dominates the system. The impedance of the whole system will then be dominated and determined by the impedance of the small measuring electrode which clearly reveals the activities of the underlying anchored cells. A model of the electrode arrangement as provided by Applied Biophysics Inc is represented in figure A.1.4. The well holds a maximum volume of about 500 μL . Figure A.1.5 shows the micrograph of the bare electrode without any cells adhered to it.

D. Electrode Inoculation

The five well gold electrodes, purchased from Applied Biophysics (Troy NY), were coated with fibronectin (BD Biosciences) using a $100 \mu\text{g mL}^{-1}$ solution that was prepared by thawing a frozen $40 \mu\text{L}$ aliquot of the 1 mg mL^{-1} fibronectin stock and adding $360 \mu\text{L}$ of sterilized PBS containing Ca^{2+} and Mg^{2+} . Endothelial cells, grown to confluence in a 100 mm culture dish, were trypsinized using 0.05% trypsin (Gibco BRL) and counted using a hemocytometer. The cells were then spun down, the trypsin drawn off, and then resuspended in 10 mL M199. From this cell solution, $400 \mu\text{L}$ were added to the electrode wells maintaining a seeding density of approximately $10^5 \text{ cells cm}^{-2}$. Endothelial cells were permitted to attach for sixteen hours in an incubator. Pictures of each well were taken before and after cell seeding. The entire surface of each well was carefully examined for endothelial cell confluence and cobblestone morphology as shown in the figure A.1.6 (a).

E. Lock-in Amplifier

The lock-in amplifier is a device which is used to detect and measure very small AC signals down to the level of a nanovolt. The greatness of the system lies in the accurate measurement of the small signal which can be buried in a noise signal thousands of times larger than the original signal of interest. The amplifier uses a technique known as the phase-sensitive detection which singles out the signal at a specific reference frequency and phase while the noise signals, which are at a different frequency are rejected and thus does not contribute to the measurement. Even when the output of the instrument reads

full scale the input sensitivity of the lock-in amplifier is achieved by adjusting the gain of the signal path. The input circuits are devised in such a way that they can handle signals many times larger than the calibrated value and thus makes a reliable measurement possible. This performance feature is called "dynamic reserve" and is a measure of the ability of a lock-in amplifier to recover signals that are buried in noise. A typical block diagram of a lock-in amplifier is shown in figure A.1.7. The following equations can give an insight into the operation of the lock-in amplifier. Assume that the input signal after amplification is represented as follows.

$$v_o A \cos \omega_o t$$

where ω_o is the reference frequency and A is the gain of the amplifier. Now the reference signal is passed through a phase shifter which alters the phase of the signal which can be represented as

$$\cos (\omega_o t + \gamma)$$

This phase-shifted signal is then passed on to the mixer circuit where it is mixed with the amplified signal of interest. The output from the mixer can be represented as follows.

$$v_o A \cos \omega_o t \cos (\omega_o t + \gamma)$$

Using trigonometric identities, the above equation can be rewritten as follows.

$$\{v_o A [\cos (2\omega_o t + \gamma) + \cos \gamma]\} / 2$$

As we can see from the above equation, the mixer output contains a high frequency component of $2\omega_0$ and a DC component, which is then passed through a low pass filter which filters out the high frequency component and allows the DC signal to pass through. The output of the low pass filter is finally read out as

$$v_o A \cos \gamma/2$$

It can be seen from the above equation that the magnitude of the signal is determined by the phase angle. Thus the lock-in amplifier has proved to be one of the most sensitive techniques for the detection of small AC signals. These amplifiers also are fitted with an internal oscillator so as to produce AC signals for external circuitry. The Lock-in amplifier used in our experiment was used for the dual purpose of providing the AC excitation to the cells as well as detecting its response.

F. Data Acquisition System

The Data Acquisition System (DAQ) for this investigation is devised using the National Instruments Signal Conditioning eXtensions for Instrumentation (SCXI) DAQ package. The system essentially consists of a chassis which acts as the base of the acquisition setup, the module which acts as the signal conditioning circuit, the terminal block which act as connectors for I/O signals to the system and a plug-in DAQ device. The plug-in DAQ device can be replaced with a module which has an inbuilt DAQ card. The chassis is highly rugged and made of aluminum. It contains an analog bus, a digital bus and a chassis controller that regulates bus operation. The analog bus transfers all the analog

signals at the input modules to the DAQ device while the digital bus is controlled by the DAQ device for chassis operation. The modules are the heart of the DAQ system as they perform the important function of signal conditioning. The module acts as a multiplexer, amplifier, filter and can also isolate voltage and current signals. There are different types of modules available and the choice depends on the application. The terminal block are attachments to the front of the module and act as a convenient interface for connecting I/O signals to the system. There are two types of terminal blocks, the direct-mount block and the terminal block extensions. The former, as the name suggests are directly connected to the module using connector screws while the latter is connected to the module using shielded cables. The DAQ system SCXI 1000 is shown in figure A.1.8. The DAQ system for this investigation has used the SCXI-1140 module housed in a SCXI 1000 chassis. The terminal block used is a SCXI-1331 which connects the input from the lock-in amplifier and passes on to the module which switches the signal between the five electrode arrays to excite the cells. The response is then detected by the lock-in amplifier and passed on to the terminal block which routes it through the module to the DAQ board on the computer. The data acquisition software collects this data from the DAQ board and stores for further analysis.

G. Impedance Biosensor Setup

The Instrumentation of the biosensor is based on the ECIS technology, which follows the technique of measuring the change in impedance of a small electrode to AC current flow. The cardinal part of the instrumentation is the specially engineered slide which has a series of wells suitable for cell culture and an array of gold electrodes that forms the base

of the slide which bridges the electronics of the biosensor setup to the individual wells. The cells are deposited on these wells which are pretreated with an adherence promoting protein called fibronectin. These cells form a monolayer over the electrodes as they settle down. A micrograph showing the endothelial cell adhesion over these electrodes is shown in figure A.1.9. A small non invasive current of about $1\ \mu\text{A}$ is passed through the gold electrodes, which results in a voltage across the electrodes oscillating at the same frequency as the applied current. The Lock-in amplifier was used to provide a non-invasive current of $1\ \mu\text{A}$ through the electrodes by applying an ac voltage of 1V through a series resistance of 1Mohm so as to excite the cells in the electrode wells. The Lock-in amplifier is also used for the phase-sensitive detection of the resulting voltage across the electrodes using the principle as discussed in the previous sections. The data was acquired for a wide range of frequencies starting from $10\ \text{Hz}$ to $10^5\ \text{Hz}$. The lock-in voltage source impedance, Z_s , was found to be around $50\ \Omega$ and the input impedance, Z_v , of the phase sensitive detector had resistive, R_v , and capacitive, C_v , components of $10\ \text{M}\Omega$ and $25\ \text{pF}$, respectively. To evaluate the α , R_b and C_m model parameters, that characterize the endothelial cell barrier function, it was necessary to convert the voltage measurements into corresponding impedance values. The constant current or current clamp, approximation has been the traditional approximation used in the ECIS method. To reduce systematic errors, voltage measurement conversion to equivalent impedances in this study included the effects of parasitic lead impedances and electrode loading. A block diagram to provide an overview of the system is presented in figure A.1.10 (a) & (b) and the instrumentation setup in the lab is shown in figure A.1.11.

The working of the biosensor setup is based on the theory that the change in the morphological characteristics of the cell is translated as the change in the measured impedance. As the cells settle down on the naked electrode, it forms a monolayer on top of the electrode. The measured impedance reflects the adhesion of the cells to the electrode surface. The barrier function associated with the endothelial monolayer restricts the current flow and we can see a marked increase in the measured resistance in the course of time during which the cells reach confluence over the electrode. The difference between the impedance measured with a naked electrode and that with the confluent layer of cells can be seen by normalizing the data. After the cells reach confluence over the electrode, the steep change in the measured data is reduced to small fluctuations that can be measured over time. These fluctuations can be attributed to the micromotion of the cells over the electrode surface. This arrangement thus serves as a basis from where we can subject the cells to various external stimuli and signals and monitor its response.

III. Results and Discussions

One of the most direct measurements of this biosensor is that of the attachment and spreading behaviors of cells. These measurements allow us to study and quantify the interaction of cultured cells with extracellular matrix (ECM) proteins and other external stimuli continuously and in real time. As mentioned in the previous section, the instrumentation is controlled by programming in LabVIEW, a scientific virtual instrumentation tool offered by National Instruments. The electrodes are first coated with fibronectin, a protein used for promoting cell adhesion and the experiment is then setup so as to run a scan of the electrodes with the M199 growth medium without any cells so

as to follow the performance of the electrodes. The scan was run over a wide range of frequencies starting from 10 Hz to a 100 KHz and the response of the naked electrode was documented using the DAQ system integrated with the computer. The sampling frequency and time were adjusted depending upon the number of frequencies used to scan and the number of wells in the electrode arrays under investigation.

The experiment is usually started with a naked electrode scan to ensure that the electrodes were in proper condition. This step is necessary as we found the electrodes were subjected to manufacturing defects like improper electrode well seal and finger prints being deposited on the electrode film which leads to erroneous data. The leads were also checked so as to make the measurement as accurate as possible. A frequency scan was run with the naked electrode filled with the growth media alone for a range of frequencies logarithmically spaced between 10 Hz and a 100000 Hz. After completing the naked scan, the electrodes were inoculated with PPAEC as discussed in the previous section. Around 400 μ L of cells suspended in the M199 medium were filled in each of the electrode well except the last well which was designated to act as a control. Figure A.1.12 shows the resistance and capacitance of the naked electrode and the cell covered electrode as a function of the scan frequency after the first scan with the cell covered electrode. The data obtained from the naked frequency scan is represented in white and the cell covered scan is represented in red. The data obtained after about 25 scans is shown in figure A.1.13 and it can be seen that the cell effect is highly reflected by the measured resistance and capacitance at a frequency range of 1-10 KHz especially around the 5 KHz range. The resistance of the cell covered electrode is as much as four times the

naked electrode resistance after the cells have attained confluence over the electrode and it is represented in the impedance graph obtained after 50 scans as shown in figure A.1.14. The normalized graph shows the increase in impedance due to the presence of the cells on top of the naked electrode and is represented in figure A.1.15.

A time series of the obtained data for naked and cell covered electrodes is presented in figure A.1.16. The stimulating frequency for this time series data was chosen to be 5.62 KHz, a log frequency around the 5 KHz range where the cell effect can be seen clearly. The steep increase in the measured resistance for the initial part of the time axis can be attributed to the adhesion of the cells on to the electrode as they settle down. It can be observed that the increase in the measured resistance ceases at a point of time which can be related to the electrode being confluent with cells and the acquired resistance from that point is limited to minor fluctuations as we move along the time axis. For a confluent electrode the fluctuations can no more be ascribed to the variation in the number of cells that cover the electrode. These fluctuations are attributed to the changes in cell shape at the nano level and the micro motion of the cells which affects the ventral spacing parameter α and the resistance between the cells R_B . The variance of these two parameters affects the measured impedance which results in the fluctuations observed. Even though it is difficult at hindsight to determine whether it is α or R_B which causes the fluctuations, studies show that both parameters are equally important in this consideration [14]. This arrangement holds well for following the cellular activity for up to 70 -80 hours accurately but studies show that the capacitance increases at low-frequency for long term use by 5-10% which may lead to a decrease in the accuracy of

observed data in the sense that the data acquired may not satisfactorily represent the state of the cells as accurately as it did for initial scans. The results shown in the figures were obtained from scans run for about 25-30 hours and hence the capacitance effect was negligible.

IV. Conclusions

The results obtained showcase the potential use and acute sensitivity of this biosensor to monitor intricate cellular activity over a considerable period of time. The data from the biosensor carries sensitive information such as the micro motion and cell adhesion coefficient which cannot be observed with the laboratory microscope. Studies have shown that the fluctuations can be safely attributed to the living cells and not artifacts as the amplifier magnification we are dealing is very less [14, 31]. It has been demonstrated with this biosensor that it is possible to stimulate a single cell to different external stimuli and follow its response over a period of time. Thus, this arrangement with its inherent simplicity and sensitive measurements holds a lot of promise to study the complex intracellular activities as will be shown in the latter parts of the thesis.

PART III

Real-Time Monitoring and Quantification of the effect of Cytochalasin D on PPAEC using the Cellular Impedance Biosensor

ABSTRACT

This section describes a technique based on the cellular impedance biosensor to study the effect of the actin disrupting drug, cytochalasin, on the Porcine Pulmonary Artery Endothelial Cells (PPAEC) at different concentrations and to examine the time-response at various levels of concentration. The behavior of these cells under the aforesaid conditions is determined by measuring the electrical resistance and the corresponding changes under different conditions. The measurements are made using the cellular impedance biosensor setup discussed in the previous section and the drug induced changes in the motility, cytoskeleton bonding and spreading of the cells over the electrode surface are measured by directly calibrating the change in resistance as a function of time. The time response of the cells to different concentrations of the drug is also calculated. The concentration of the drug injected to the cell culture varied from zero concentration in the first well to a lethal dose at the penultimate well. The last well contained only cells and was not exposed to the drug so as to act as a control. The cell response for these varying concentrations gave satisfying results and we found that the drug effect saturated over a particular concentration that gives an idea about the receptors of the cells. A sample for microscopy was prepared from the same cell culture and stained for actin filaments before adding the drug in similar concentrations. The sample was viewed under a laser confocal microscope and the images obtained were used to quantify the data from the biosensor. A wavelet transformation algorithm was applied to the acquired data so as to capture events and trends induced by the drug. The result of these experiments throws light on the cell behavior under different levels of toxic media

and provides a base for analyzing the cell behavior under different ions and chemical compounds which drives the research closer to the physiological scale.

I. Introduction

The Properties of mammalian endothelial cells and their behavior under different conditions is highly interesting and calls for an in depth analysis of is important both in a scientific and medical point of view. As discussed in part I of this thesis, the cytoskeleton stands out as one of the most important parts of the cell. It is an important, complex, and dynamic cell component which maintains the cell's shape, anchors the cell organelles and performs a key function in the process of cell growth, motility and cell-substrate adhesion. The actin filaments are the most abundant of all cytoskeletal proteins and this part of the thesis is dedicated to show the use of the biosensor in analyzing the actin response to an inhibitor at different levels which would provide a model of intracellular micromechanics under pathological or unfavorable conditions.

Actin was arguably discovered by Straub in 1942 [47]. It is found to be a widely expressed protein in nearly all kinds of eukaryotic cells and it is the major supporting factor for the complex ultrastructure of the cell and its many motions. Optical techniques have revealed the size of the filaments to be around 8 nm and being the thinnest of all the cytoskeletal proteins, they carry the name microfilaments. The actin filament is a rare combination of strength and sensitivity in the sense that they are used in many of the strenuous structural tasks, but are also easily and continuously disassembled. The hallmark of the actin filament is this dynamic character by which they continuously assemble and disassemble as the needs of the cell change from one movement to another. It can be imagined as a filament that grows at one end and dissolves at the other, so that the whole structure slowly steps through the cell without getting any longer or shorter. It

has been shown that the cells form and maintain their shape and structure depending upon the integrity of the actin cytoskeleton [48-50]. The study also mentions that the actin is highly essential in the cell motility during normal and pathophysiological processes. Signal transduction is an important function in cellular life and it has been shown that it is dependent on the dynamic remodeling of the cytoskeleton during cellular processes which in turn changes the cell shape and motility in response to external stimuli [48]. The number of investigations and studies show the relevance and importance of the actin filaments in the motile activities of the cell. This dynamic aspect of the actin makes it a valuable asset to all the cellular activities and hence the actin actively pursued by cell biologists world over with an aim to understand the cellular micromechanics so as to predict the cellular behavior under hostile and pathological conditions. The study of the cellular micromechanics thus is a valuable tool in drug development and tissue engineering

This section of the thesis demonstrates the use of the cellular biosensor setup to monitor the change in the actin cytoskeleton in response to unfavorable drugs and follow the reaction kinetics of the drug as a function of the concentration. The optical technique used to quantify the results from the impedance biosensor is detailed along with the protocol used for the actin filament staining. A wavelet analysis is formulated by dynamically linking the NI LabVIEW virtual instrumentation tool with Matlab to provide a time-frequency representation. The optical technique is used to quantify the actin filament disruption with respect to the concentration of drug added so that a direct correlation can be made with the acquired data. The wavelet analysis is used to track the

events that occur during normal cellular adhesion to the microelectrode and those that follow the drug addition. This work is thus aimed to device a real-time measurement package would give a new dimension to monitor cells under hostile conditions, with an aim to provide useful applications in the artificial tissue engineering processes and drug discovery methods.

II. Methods and Materials

A. Cell Culture and Actin Cytotoxicity Assay

Endothelial Cells were grown at an ambience of 37 deg C in a humidified incubator (VWR) with a constant level of 5% CO₂ for pH and humidity control. The medium used for the cell culture is M199 (Gibco Inc) and is supplemented with 10% female bovine serum (FBS). Cell Suspensions were prepared using trypsin whenever there was a need to passage the cells from a single dish to multiple dishes or the cells were needed to carryout the experiments. The Cells are allowed to proliferate on pre treated culture dishes purchased from Corning Inc, New York. The Medium was carefully changed every alternate day so as to ensure the healthy growth of cells.

Cytochalasin D was chosen to induce cytotoxicity since it specifically inhibits actin polymerization. Cytochalasin basically disrupts the actin filaments of the cells and there by breaks the cellular bond to the substratum and neighboring cells. Cytochalasin is known to be membrane permeable and its addition to living cells blocks cell locomotion and cytokinesis and hence an ideal candidate to study the disruption of the actin cytoskeleton. Cytochalasin D was purchased from SIGMA, St.Louis, MO and was

broken down into aliquots in sterile micro centrifuge tubes at a concentration of 3mM. A stock solution of 30uM was obtained by dissolving 10uL of the 3mM stock in 990uL of the M199 medium. Different Concentrations of the drug varying from a lethal dose to the smallest concentration were prepared by serial dilution of the 30uM stock. The drug was mixed thoroughly into different concentrations and added to the wells which house the gold electrodes.

Actin is that part of the cytoskeleton which allows movement of cells and cellular processes. Actin is a globular protein with an ATP binding site in the center of the molecule. This serves as a site for nucleation and further growth of the actin protofilament. Polymerization of actin filaments can occur via a network regulated by filamin. This protein works like a clip to connect the filaments at the crossover points. Other proteins create bundles of actin filaments. Projection of cellular processes is made possible by actin polymerization and formation of bundles or networks. G-actin forms F-actin (the filament) in the presence of ATP, Mg and K. The concentration of G-actin is also critical. Above the critical concentration C_c of G-actin, the molecules will polymerize. Below the critical concentration, the actin filaments will depolymerize.

Cytochalasin binds to the + end of F-actin and prevents further addition of G actin thereby disrupting the actin filament. Its effect on endothelial cells is very important, as the behavior of endothelial cells under different toxic conditions is a valuable parameter to study the development of atherosclerosis

Fibronectin was the choice of protein used to pre-treat the gold electrodes and was purchased from BD Biosciences. MA. It was mixed thoroughly with 1mL of De-Ionized

water so as to prepare the coating medium. A measure of 40uL of the prepared solution was carefully added on top each of the electrode surface followed by the addition of 360uL of Phosphate Buffer Solution (PBS) to each of the wells which aids in the effective coating of the proteins on to the gold electrodes. The Mixture of PBS and Fibronectin was allowed to stay in each of the wells for about 20-30 minutes to ensure proper coating. The PBS was then removed from all the wells and replaced with 400uL of M199 Medium and allowed to equilibrate for a short period of time.

This Naked Electrode arrangement was then setup inside the incubator and the impedance of all the 10 wells used were measured as explained in the previous part to carry out a study on the electrodes. The Cell Suspensions were added to the wells with one of the wells acting as a control electrode without any cells. The impedance measurements were carried out over a period of 16 hours so as to study the changes in mobility and proliferation of the cells over the coated electrode. The drug was added after ensuring that the cells have formed a confluent layer over the electrodes and acquired data showed fluctuations corresponding to the cellular micromotion. The addition of the drug was sequenced such that the first well had the highest concentration and the subsequent wells had concentrations of the drug in decreasing order. The toxin was not added to the last well so as to act as a control for cellular behavior. All the measurements were made at a frequency of 5.62 KHz as we found that this frequency best represented the effect of attached cells and their behavior. Also we used a single frequency for measuring the effects so as to get better time response and ensure an accurate trace of the cellular response to the drug.

B. Immunofluorescence of the Actin Cytoskeleton.

The Immunofluorescence technique has proved to be one of the most efficient ways to look at the cell and its components. It is possible that we can visualize specific parts of the cell using this method. We used this technique as a means to quantify the cellular response to the drug obtained from the biosensor. The same culture of cells was used to inoculate the electrodes of the biosensor and also prepare the sample for confocal microscopy. This was done so as to correlate the data from the biosensor with the images which would reflect the physical happenings inside the cell. The Immunofluorescence technique consisted of four important procedures namely fixation, permeabilization, blocking and staining.

Fixation is the first and foremost procedure which preserves the cellular ultrastructure and antigenic protein sites. The main intent of the fixation procedure is to maintain the original morphology of the cell. There are a plethora of commercially available fixative agents and the choice of the agent depends upon the protein of interest. Formaldehyde is known to preserve actin well and hence was used as the fixation agent in our case. After proper fixation of the cells, it was necessary to permeabilize the cells. The goal of this procedure is to permeabilize the plasma membrane so as to make it accessible to the stains and anti-bodies used in the procedure. It is important to block other potential protein-binding sites which would result in non-specific binding. A blocking agent is used to perform this task that does not react with the anti-bodies used, but reacts with the

other potential binding proteins. In this study, Triton-X and BSA were used in appropriate concentrations as the permeabilization agent and blocking agent respectively and satisfactory results were obtained. After the above mentioned procedures, the cells were stained for the actin filaments using Phalloidin, commercially available as texas red dye. On completion of the staining procedure, the sample was taken to the confocal microscopy facility and the imaging session was carried out. A detailed protocol of the staining procedure is outlined in the appendix column.

C. Laser Confocal Microscopy

Visualizing the cell is a cardinal part of cell biology and plays an important role in quantifying the results in our case. We used the laser confocal microscopy to view the cells which were stained for the different filamentous proteins of the cytoskeleton. In this part of the thesis, the detailed protocol for the staining of actin filaments and visualizing it under the confocal microscope is discussed. As far as the small world of the cell is concerned, the confocal microscopy is viewed as a major advancement over the traditional light microscope in the sense that it allows us to visualize deep within cells and tissues and also make three-dimensional slices of the specimen under interest [51].

The term “confocal” is used to describe the arrangement wherein a single point on the sample is focused by two lenses. The Major advantage of the confocal microscope over its traditional counterpart is the presence of an appropriately positioned confocal pinhole shown in figure A.1.18 (a), which will pass light only from the plane of focus and light from the out of focus planes are blocked and not allowed to reach the detector. This

principle helps in obtaining images of exceptional resolution and clarity, and presents the user an option to collect optical slices of the sample so as to create a 3D representation of the specimen by changing the level or plane at which the sample is observed. As the plane of focus is changed, we move through the vertical section of the sample and thus a series of X-Y images are obtained for different Z values. This series of images would give a three-dimensional representation of the object as shown in figure A.1.18 (b).

The practical confocal microscope is usually combined with a scanning system that uses a laser light source. The image is built by scanning a point of laser light across the sample in X and Y directions. The detector used to detect the reflected light in the case of a laser scanning system is generally a photomultiplier tube. The signal from the photomultiplier tube is converted to a digital form that contains information on the position of the laser in the image and the amount of light coming from the sample. A computer interfaced with the confocal microscopy is used to store the intensity value of each point which is contained in the photomultiplier signal and presents these in the appropriate order on a high-resolution video monitor to display the image. To collect a series of images, the focus is shifted by a fixed amount by the software controls and the object is scanned again to produce the next image at the different Z position. This image is stored and the process repeated to build the 3D data set. A typical confocal system is represented in figure A.1.17.

Confocal microscopes possess several advantages over conventional microscopy. Primarily, the resolution of the confocal microscopes are about 1.5 times larger than the conventional microscopes and they also have a higher level of sensitivity compared to

conventional microscopes due to highly sensitive light detectors and the ability to accumulate images captured over time. As mentioned above, another key feature of the confocal microscope is the ability to digitally reconstruct 3D representations of the sample.

Confocal microscopy is now being used in a large variety of scientific fields [52-53] where it is used to analyze subcellular functions, such as pH gradients and membrane potentials, using specific fluorescent dyes and to measure intracellular changes in ion concentrations of molecules such as calcium, sodium and magnesium. This microscopy technique has thus generated a tremendous amount of excitement in the research community. This technique has rapidly become the technique of choice for researchers, particularly in the biosciences. With the recent availability of more affordable confocal and two-photon microscopes, this technology holds a great deal of potential for allowing the visualization of the cellular world.

With its cutting edge technology, unique advantages and the fact that general staining protocols works well with the confocal microscope, it was an obvious choice for our investigation and it proved to be a valuable tool in quantifying the data obtained from the biosensor as will be discussed later.

D. Wavelet Transforms

The term **wavelet** means a **small wave**. Wavelet transforms have played an increasingly important role in clinical and medical signal analysis over the recent years. It has been applied to a wide variety of biomedical signals including the ECG, EEG, EMG, MRI data, heart beats, and DNA sequences. The versatility of the wavelet analysis in capturing the high frequency events and trends in the signal has made it a valuable tool in our study to investigate the acquired signal.

Wavelets are often termed as the twentieth century Fourier transform and are found to be best suited for analyzing signals which are described as noisy, transient and so on [54]. The strong point of the wavelet analysis is its distinct ability to examine the signal simultaneously in time and frequency domains. Even though the basic concept behind the wavelet analysis has its roots to the early part of the twentieth century [Alfred Haar formed the first wavelet function in 1909], it was not until the mid 1980s that it was recognized as an efficient tool for time-frequency analysis. Many eminent investigators like Drs. Ingrid Daubechies, Daniel Kleppner, Stéphane Mallat and Yves Meyer contributed to the transformation of wavelets from being a theoretical concept to a practical tool. A simple yet efficient description of the wavelets as given by the above mentioned investigators is quoted below for the understanding of the reader.

“One way of thinking about wavelets is to consider how our eyes look at the world. In the real world, you can observe a forest like the one shown in the photograph on the next page from many vantage points—in effect, at different scales of resolution. From the

window of a cross-country jet, for example, the forest appears to be a solid canopy of green. From the window of an automobile on the ground, the canopy resolves into individual trees, and if you get out of the car and move closer, you begin to see branches and leaves. If you then pull out a magnifying glass, you might find a drop of dew at the end of a leaf. As you zoom in at smaller and smaller scales, you can find details that you didn't see before”.

The wavelet transform can be explained by the simple equation as explained by Dr.Polikar [55]. For a given function $x(t)$, the wavelet transformation can be represented as follows.

$$CWT_x^\psi(\tau, s) = \Psi_x^\psi(\tau, s) = \frac{1}{\sqrt{|s|}} \int x(t) \psi^* \left(\frac{t - \tau}{s} \right) dt$$

As seen in the above equation, the transformed signal is a function of two variables, τ and s , the **translation** and **scale** parameters, respectively. $\psi(t)$ is the transforming function, and it is called **the mother wavelet**.

The term mother is used in the sense that the different functions used in the transformation process are derived from one main function, which is the mother wavelet. In other words, the mother wavelet is a prototype for generating the other window functions. The term translation is used here to describe the location of the window, as the window is shifted or translated throughout the signal and corresponds to the time axis in the actual wavelet transform. The term s is the scale which can literally be viewed as the inverse of frequency and corresponds to the contraction and dilation of the mother

wavelet. This parameter in the wavelet analysis is similar to the scale used in geographical maps in the sense that high scales correspond to a holistic view of the signal with minimum of detail and low scales correspond to a local and highly detailed view. Similarly, low frequencies (High Scales) correspond to a global view of the signal usually covering the entire signal, whereas high frequencies (low scales) correspond to a detailed information usually lasting for a relatively short time. The mother wavelet is translated throughout the signal at different scales to capture different trends and thus forms the essence of multi-resolution analysis.

A Graphical program was developed in LabVIEW to apply time-frequency analysis to the acquired data. The provision available in LabVIEW to dynamically link with Matlab has been used to our advantage to present a neat wavelet transformation algorithm. The In-built functions in matlab calculate the wavelet coefficients according to the scale and mother wavelet used and we get a 3D representation of the transformed data. The wavelet transformation is applied in an attempt to capture the cellular fluctuations and compare the cellular behavior before and after the addition of the drug.

III. Results and Discussions

It was necessary to ensure the proper treatment of fibronectin to the gold electrodes so that we get a good cellular binding to the electrode. Any compromise in this may lead to erroneous results with regard to predicting the behavior of these cells under different conditions since the spreading, morphology and cell motility depend upon the good attach characteristics of the cells to the measuring electrode. The Resistance of the naked

gold electrodes was ~2k (Ranging from 1.6-2k). When a 400uL suspension of cultured endothelial cells were added to each of the dishes, the resistance measured gradually increased as the cells started to form a monolayer over the gold electrode as explained in the previous section. The measured resistance peaked to value around 9-10K. The observed data dwindled down to minor fluctuations upon the cells attaining confluence over the electrode and these fluctuations can be attributed to the micro motion of the cells over the electrode surface. All the wells indicated a consistent attach pattern in the various experiments that were carried out. The cells were allowed to attach overnight to ensure a good confluent layer over the electrode and the impedance measurements were carried out through the Phase Lock-in Amplifier and the data was recorded using the Virtual Instrumentation software Lab VIEW.

Cytochalasin was added to each of the wells in different concentrations ranging from a highest concentration of 3.16uM in the first well to a least of 0.316uM in the last well after allowing the cells to attach overnight. We started with a wide range of concentrations of the drug, from a lethal dose of 30uM to a minimal concentration of 0.003uM. The pattern of the data acquired with these concentrations was discontinuous as shown in the figures A.1.20 and A.1.21. We also found a saturation effect above a concentration of 3uM and below a concentration of 0.3uM. After repeated trials we found that a concentration range of 3uM-0.3uM would give us a regularly spaced and meaningful data, which resulted in logarithmically spaced concentrations of 3.16uM-0.316uM. The well with the highest concentration of the drug recorded the maximum

drop in resistance and the subsequent wells recorded a drop proportional to the drug concentration in the first few minutes.

The scan was run at a single frequency of 5.62 KHz so as to facilitate the continuous tracking of cellular response to the drug and also for the reason that cellular activity was best captured at this frequency. The cells treated with the highest concentration recorded an immediate drop in the measured impedance indicating the degree of inflicted damage by the drug. A suitable explanation for the drop in the impedance can be attributed to the degree of disruption of the actin filaments which should sever the cell from the substratum, allowing a free path for the current flow. Figure A.1.22 and A.1.23 represent the acquired data for each of the drug concentration and it can be clearly seen that the drop in the measured value is clearly correlated with the drug concentration. Figure A.1.24 is provided to obtain a better understanding of the drug effect on the measured impedance.

Cells from the same culture as those used for impedance studies were used to prepare a sample for laser confocal microscopy. The cells were stained for actin filaments and subjected to the same concentrations of cytochalasin before fixing them as explained in the Immunofluorescence procedure. These samples provided a clear picture, when viewed under the confocal microscope, of what actually happened inside the cell. The different concentrations of the drug resulted in different degree of disruption of the actin filaments, which was clearly evident from the acquired images. These images bear a direct correlation with the acquired data, which shows the efficiency of the biosensor in tracking the intracellular activities. Figure A.1.25 show the actin cytoskeleton of the

endothelial cells before inducing it with the drug and figure A.1.26 shows a series of images which represent the actin disruption as a function of the drug concentration. The acquired images were processed using the Matlab image processing toolbox and the effective area covered by the cell and its filaments was obtained as a function of the drug concentration. The matlab code is presented in the appendix and figure A.1.27 shows the cell area vs. the drug concentration graph, which gives a clear idea about the degree of disruption of the filaments for the different drug concentrations.

A wavelet transformation algorithm was applied to the acquired data with an aim to capture the fluctuations due to cellular micromotion and the effects due to addition of the drug. The wavelet program is coded in a combination of LabVIEW-Matlab environment so as to use the best of both tools. The wavelet analysis is used here so as to capture events which are reflected as varying frequency components in the acquired signal along with the temporal information. A wavelet transform representation for different strengths of the drug is shown as a series of graphs in figure A.1.28. The figures from top to bottom in A.1.28 represent the wavelet transform for concentrations of 3.16 μM , 1.78 μM , 0.562 μM and the attach scan. The area markers A-F are presented for easy interpretation of the results. The area A in the first figure represents absolutely no fluctuations that correspond to cellular activity which signals cell death. The wavelet graph for the 1.78 μM data represents very small fluctuations indicated by area markers B-D. These events cannot be interpreted from the original data and this is where the worth of a signal processing method such as wavelets is proved. Even though the acquired data for the 0.562 μM concentration of drug and the attach scan does not reveal

a great deal of difference, the wavelet analysis clearly shows the difference in the cellular behavior. The markers E and F can be used to identify this difference in the sense that the attach scan has more power in the wavelet transform which reflects the fact that the cells which were treated with 0.562 μM of the drug were less motile than the normal cells. This can be an important observation and the wavelet transform captures these kind of trends and events not visible to the naked eye. We have high values for the wavelet coefficients at a particular scale and time if the correlation between the mother wavelet and the signal at that instance is high and vice-versa. Thus the use of different scales throughout the signal gives good time resolution and poor frequency resolution at high frequencies and good frequency resolution and poor time resolution at low frequencies.

As we can see from the graphical representation of the results and images, an acute concentration of the drug made the actin filaments of the cells disassemble instantly while the cells were still able to attach and spread for lower concentrations of the drug. But as the length of time of drug exposure increased the micromotion of the cells as reflected by the measured fluctuations gradually begun to die down. The final well of the electrode array was used as a control where no drug was added to ensure that the cells were not affected by other sources.

IV. Conclusions

Different Drugs attack or alter different but specific parts of the cell such as the Cytochalasin disrupts the actin filaments. These Cytotoxicity tests give a deep insight in to the mechanism of these cells and how the various structures of the cell contribute in its division, proliferation and migration.

This dynamic response of the cells to different environments and sensitivity to toxicants is a valuable tool for many drug discovery methods and tissue engineering applications. These impedance measurements are a definitive indicator for cell growth, proliferation and its sensitivity to different external stimuli and this section has demonstrated its ability to conduct specific studies on different intracellular components. Different imaging modalities can be used to study the cytoskeleton mechanism by staining the cells for the required structure. The relevant optical technique combined with the impedance biosensor thus promises to be an efficient tool in studying the various behavioral characteristics of cells.

Part IV

Real-Time Monitoring of Microtubule Disruption using the Biosensor Setup

ABSTRACT

Microtubules are the one of the most important filaments of the cytoskeleton along with the actin and intermediate filaments. The microtubules work alone or along with the other cytoskeletal filaments and play a crucial role in various cellular processes such as genetic material segregation, intracellular transport, signal transduction and maintenance of cell shape. Recent drug discovery methods are focused primarily on monitoring the response of the microtubules as they essentially control the cell and its motile activities. Interaction of different drugs with the microtubules and analyzing its response holds a lot of promise for effective development of anti-cancer and anti-mitosis drugs. Studies have shown the importance of developing highly selective drugs which target the microtubules of the cancerous cells and essentially bring all the motile activities to a cessation. In this case, the cellular impedance biosensor proves to be a useful tool in analyzing the cellular response to microtubule specific drugs. As in the previous section, the cells are exposed to different concentrations of a microtubule inhibiting drug and its response is captured by the impedance biosensor. The data is then quantified through the imaging procedure discussed in the previous chapter and a similar wavelet analysis is applied to the acquired data so as to capture the trends and events. This method holds good potential to be developed as an effective method to screen different drugs by analyzing the electrical response and putting the acquired signal through rigorous quantification procedures.

I. Introduction

It was in the 1950's that the microtubules were first observed when investigators Manton, Clarke, Fawcett and Porter were conducting electron microscopy studies of cell sections. Microtubules are one of the principal components of the cytoskeleton and are rigid hollow rods approximately 25 nm in diameter. They are similar to actin filaments in the sense that they are dynamic structures that undergo continual assembly and disassembly within the cell. They perform a pivotal role in both the cell shape and in a variety of cell movements, including some forms of cell locomotion, the intracellular transport, and the separation of chromosomes during mitosis. Microtubules are composed of a single type of globular protein, called tubulin. Tubulin dimers polymerize to form microtubules, which generally consist of 13 linear protofilaments assembled around a hollow core as shown in figure A.1.6 (c). The protofilaments, which are composed of head-to-tail arrays of tubulin dimers, are arranged in parallel. Consequently, microtubules (like actin filaments) are polar structures with two distinct ends: a fast-growing plus end and a slow-growing minus end. This polarity is an important consideration in determining the direction of movement along microtubules, just as the polarity of actin filaments defines the direction of myosin movement. Thus the microtubules are one of the most dynamic and important proteins among the cytoskeleton family.

This versatility and dynamic nature of the microtubules, which play a crucial and cardinal role in the process of cell division and its motile activities, has made it a target of study in the drug development procedures for different types of cancer and also in Alzheimer's disease. In such pathological cases, attacking the so-called "killer cells" specifically holds

the key to an effective cure. The recent drug discovery methods have focused on targeting the microtubules of the cancerous cells. The fact that an irreversible damage of microtubules would cause cell death is used to advantage in developing treatments for diseases like cancer. It has been found that there is a trade-off in using this method as the non-specificity of the drugs result in anti-angiogenic activities by affecting the motile activities of the healthy cells surrounding the tumor cells. Recent technologies are focused on making the drugs specific to disrupt the motility of tumor cells alone and for this reason, the study of the microtubule dynamics of a cell culture population in response to different external stimuli provides a good model for studying the importance of these filamentous proteins in cellular processes.

The purpose of this chapter is to exhibit the ability of the impedance biosensor in successfully following the tubulin dynamics as in the case of actin discussed in the previous chapter. The endothelial cells are plated on to the gold electrodes and subjected to different doses of the microtubule disrupting drug. The response is monitored electrically and the acquired signal is analyzed using the National Instruments LabVIEW package. A similar optical technique as described in the previous section is applied here to view the microtubules under different conditions, but the difference here lies in the sample preparation protocol which is completely different from the previous one. A wavelet transformation is applied to the acquired impedance data so as to analyze the information in both time and frequency planes.

This ability of the biosensor in acquiring the impedance signal which reflects the microtubule dynamics in response to toxins and drugs can prove to be a valuable tool in

the development of effective therapeutic methods for different types of cancer and other relevant pathologies.

II. Methods and Materials

A. Tubulin Cytotoxicity Assay

The endothelial cells were isolated and cultured as described in the previous sections. A healthy batch of cells between the fourth and eighth passages was used in the cytotoxicity assay. The cells were plated on to the gold electrode of the biosensor and then electrically stimulated. The cells were exposed to the microtubule affecting drugs after it was made sure that they attained confluence. The cellular response to different concentration of the drugs was electrically monitored and the acquired signals were stored for further analysis as explained before.

Nocodazole was the drug used to study the microtubule dynamics. It is an anti-mitotic agent that disrupts microtubules by binding tau-tubulin and preventing formation of one of the two interchain disulfide linkages, thus inhibiting microtubule dynamics and triggering the disruption of mitotic spindle function, and fragmentation of the golgi complex. It also arrests the cell cycle at G_2/M phase and prevents phosphorylation of the T cell antigen receptors resulting in the inhibition of mitochondrial activity. It is to be noted that the action of nocodazole is distinctly different from the popular anti-cancer drug taxol. While taxol stabilizes the microtubules by triggering the polymerization of unpolymerized tubulin, nocodazole completely disrupts the tubules. The end result though in both cases is the arresting of the motile activities of the cell. Cells were plated

on to the gold electrodes and after ensuring that they have formed a confluent layer on top of the electrode surface, they are exposed to the drugs. The cells were exposed to nocodazole at concentrations of 10 μM , 5.62 μM and 3.16 μM . The cellular response to these drugs is monitored electrically for the different concentrations similar to the cytotoxicity assay in the case of the microfilaments. The same passage of cells was used to prepare a sample for the immunofluorescent studies with which the microtubules are visualized.

B. Immunofluorescence of the Microtubules

Even though the procedure is similar as staining for actin, the protocol for microtubules differs considerably in terms of the chemicals used. The protocol consists of the four important steps: fixation, permeabilization, blocking and staining. The important point to be noted in the case of microtubules is the use of two antibodies in the staining procedure. The primary antibody binds to the target, which is the tubulin here and the secondary antibody with the fluorescence in turn binds to the primary antibody thus making the microtubules clearly visible. The primary used here is a Anti-Bovine α -tubulin mouse monoclonal antibody which binds to the tubulin and the secondary is a Goat anti-mouse antibody which binds to the primary. After the staining procedure is completed, the sample is taken down to the confocal microscopy facility and imaged. A good fixation should yield an image where the microtubule spindles can be individually seen.

III. Results and Discussions

The tubulin cytotoxicity assay was carried out in a similar manner as to that of the microfilaments. The electrodes were first subjected to a frequency scan over a range of 10 Hz-100 KHz so as to ensure that they are in proper condition. After the initial scans and the formation of a cellular monolayer over the measuring electrode, nocodazole was added to the wells in different concentrations. We started with concentrations of 100 μ M, 56.2 μ M, 31.6 μ M and 10 μ M. These were the initial concentrations that we tried for determining the cellular response and also to zero in on the critical concentrations where in we get varying cellular response. As we can see from the figure A.1.29, we got a similar cellular response for all concentrations above 10 μ M and this gives an idea about the dose dependence of the cellular response to drugs. After the initial studies we got varying cellular response for concentrations of 10 μ M, 5.62 μ M and 3.16 μ M of nocodazole. Figure A.1.30 gives the series of acquired data for the as mentioned above and the difference in the cellular response can be clearly seen for the different concentrations of the drug. An all-in one graph is shown in figure A.1.31 which would be helpful for the reader to get a better understanding of the reaction kinetics of nocodazole at different concentrations.

The ability of the biosensor in following the dynamics of different cytoskeletal elements is elucidated in figure A.1.33, where the data acquired from the actin and tubulin cytotoxicity assays are compared at the critical concentration of 3.16 μ M. It can clearly be seen that there is a completely cessation of cellular activity when treated with

cytochalasin D as opposed to a highly evident cellular activity in the data acquired after treatment with nocodazole at the same concentration. This comparison is highly helpful in determining the importance of the different cytoskeletal elements and the conditions under which they fail.

The wavelet transformation is also applied for the tubulin cytotoxicity assay in an attempt to see the fluctuations that correspond to the cellular micromotion. A wavelet transformation algorithm as explained before is applied to the data acquired at different concentrations of the drug and compared to the attach scan. Figure A.1.32 represents the series of wavelet transformed data for the different concentrations of nocodazole. The immunofluorescence staining of the tubulin was also carried out in order to visualize the microtubules. Figure A.1.34 represents the series of images acquired from the confocal microscope. It can be seen that the staining does not reveal a whole lot of microtubules yet gives us an idea of the tubule spindles arranged in the cytoskeleton.

IV. Conclusions

The microtubules play a crucial role in cell division and along with the microfilaments help the cell in locomotion and other motile activities. The sensitivity of the biosensor in following the tubule dynamics and capture the varying cellular response for different drugs has been demonstrated. This setup when combined with the relevant optical techniques and signal processing algorithms can make a very good package for *in vitro* toxicology monitoring and other cell based assays.

The development of effective anti-mitotic and anti-cancer drugs is very important for treatment of different types of cancers and other cell-based pathologies. The ability of this simple biosensor setup to effectively monitor the microtubule dynamics under the influence of toxins and drugs provides valuable information in drug discovery research.

Part V

Future Considerations and Conclusions

I. Introduction

The intent of this master's thesis has been to present the impedance biosensor as a potential tool in detecting the motile activities of the cell and tracking the intracellular response to different drugs and pharmaceutical agents. Part I served as an introduction to the history behind electrophysiology and the various methods to stimulate biological specimens electrically and also to analyze their electrical response. The work pioneered by Drs. Giaver and Keese, which forms the basis of this research and other important investigations pertaining to this study were discussed which formed a good foundation for understanding the principle behind this thesis. The focus in part II shifted to discussing and proving the ability of the biosensor arrangement to follow cellular behavior using the impedance measurements and showcased the ability of the biosensor to potentially monitor the intracellular dynamics in response to external stimuli. This potential was realized in parts III and IV when the biosensor was combined with relevant optical techniques and signal processing algorithms to successfully monitor the cytoskeleton response to drugs and quantify the same. The purpose of this chapter is to give a conclusion to this work and discuss the plausible modifications to the biosensor setup and explore the abilities of this arrangement to extend its applications beyond the aspects already discussed in this thesis.

II. Future Considerations

A. Real-time Monitoring of Intermediate Filament Dynamics

The immediate future application of this biosensor would be to capture the intracellular mechanics of the other important cytoskeletal element, the intermediate filament. Intermediate filaments are one of three types of cytoskeletal elements which frequently work together with the other two components to enhance structural integrity, cell shape, and cell and organelle motility. Intermediate filaments range in diameter from 8-10 nm and are mechanically stable and durable. They are the most insoluble part of the cell and are found mostly in cell types that are designed to withstand mechanical stress. The intermediate filaments differ from the other cytoskeletal proteins in the manner that they are classified in to five different types across different kind of cells. Types I and II filaments are acidic Keratin and basic Keratin, respectively which are produced by different types of epithelial cells. The type III Intermediate filaments are distributed in a number of cell types including Vimentin in fibroblasts, endothelial cells and leukocytes, desmin in muscle etc. Type IV is "internexin" found in lens fibers of the eye (filensin and phakinin). Type V is the lamin which has a nuclear signal sequence so they can form a filamentous support inside the inner nuclear membrane. Lamins are vital to the re-formation of the nuclear envelope after cell division. Thus the Intermediate filaments perform a vital role as the other cytoskeletal proteins in the motile activities of the cell and similar study on them as in the case of actin and microtubules can be carried out using the impedance biosensor with high sensitivity as discussed in the previous sections of the thesis.

B. Monitoring Cellular Behavior on Artificial Surfaces

Cellular interaction and their motile behavior on artificial surfaces perform a key role in cell therapy and tissue engineering. Studies in the recent times have concentrated on perfecting biomimetic surfaces that promotes cellular adhesion and acts as a scaffold for cell growth. In spite of many different potential ways to engineer artificial tissues and organs, the most appealing procedure followed is the combination of the patients own cells with the artificial scaffolds. These scaffolds deliver the cells to the patient's body, provide a space for the tissue growth and essentially control the newly engineered tissue. The most favorable artificial scaffold for cell growth has been the hydrogels because of their favorable characteristics. They have a high percentage of water and a low coefficient of friction which help them to remarkably mimic the different tissues of the body.

Perfecting the characteristics of the artificial material so as to promote effective cellular growth is in itself a separate channel of research. After successful engineering of the proposed artificial scaffold, accurate monitoring of the cellular behavior holds the key in determining the performance of the material.

The impedance biosensor discussed in this thesis potentially holds the answer to high sensitivity monitoring of cellular behavior on these hydrogel surfaces. A proposed method to monitor the cellular activity on hydrogel surfaces is discussed in the next section.

The Proposed Method: The proposed future modification to the biosensor is to combine the technology of polydimethylsiloxane (PDMS) micro-channel fabrication with the Electrode Cell-substrate Impedance Sensing (ECIS) methodology, which would result in a modified impedance sensing system that can track the motile activities of the cell over artificial surfaces. The idea here is to make the sensing electrode accessible by means of a microchannel through a layer of PDMS, which would act as a passage for depositing the pre-gel solution on top of the measuring electrode and then allowing it to be polymerized. This arrangement would thus give us a layer of hydrogel on top of the measuring electrode, to which the cells can be seeded. The PDMS layer is placed over the array of electrodes with a tight seal and may be used for walls of a chamber, which is filled with cell medium such as M199. We hypothesize that it would be possible to track the adhesion of the cells on to the hydrogel surface, its proliferation, and other induced responses by the impedance measurement system.

Micro channel fabrication of wells and a chamber: Wells and a chamber would be fabricated using PolyDiMethySiloxane (PDMS, Dow Corning Sylgard Silicone Elastomer –184, Krayden Inc., Houston, Texas) and photolithography. The figure A.1.35 illustrates general micro-fabrication sequence of the micro-channel geometry. Assumed the thickness of hydrogel layer can be controlled less than 30 μ m, SU-8 2035 is used to develop one micro-channel including five wells with 55 μ m height and 280 μ m diameter exactly to cover the working electrode and another SU-8 2100 is employed to build another micro-channel as a chamber which occupies five wells and counter electrodes, respectively. Table A.3.2 shows the different type of products available to fabricate micro

channels of different dimensions. Accordingly two types of photo-resist are needed and thus two PDMS micro-channels are built and these two channels are combined into one channel with the plasma bonding.

The proposed system uses a combination of the above mentioned technologies in a way so as to present an experimental setup which would facilitate real-time monitoring of the cell behavior on different bio-material surfaces such as different type of hydrogels. The original five-well electrode arrangement is modified by removing the plastic wells that cover the electrodes and is left with the thin base with a series of five gold micro electrodes. The plastic wells are replaced with PDMS channel which cover the measuring electrodes and form a tight seal. Fabricated is the chamber with PDMS, which is filled with culture medium (electrolyte) so as to allow current flow. Figure A.1.36 represents the proposed experimental setup. Micro channels are fabricated through PDMS blocks on to the top of the five measuring electrodes and thus making the electrodes accessible from the outside. The heights of wells and chamber are different, i.e. the chamber's height is more than that of wells. They are possibly built by controlling the spinning speed of the coater and the type of photo-resist. This arrangement can give an accurate deposition of the pre-gel solution over the exact surface of the measuring electrode. The conductivity of the proposed biomaterial should ideally be high enough so that its impedance does not overshadow the resistance measured due to the cellular activity. This can be measured experimentally and made sure that the electrical response of the hydrogel is favorable to monitor the cellular activity.

III. Part Conclusions

The ability of the biosensor to monitor the cellular motile activities *in vitro* with high sensitivity was discussed with the appropriate results in part II. The cells were carefully cultured at 37 deg C and 5 % CO₂ incubator using aseptic techniques. These cells were then transferred from the culture dishes and allowed to grow on top of a gold microelectrode. This setup was useful in stimulating the cells with a non-invasive current of 1 μ A at different frequencies and analyzing the electrical response of the cells. It was shown that the measured impedance reflected the cellular behavior on top of the electrode accurately. This ability of the biosensor was extended to monitor the intracellular dynamics in response to different drugs which is discussed in part III and IV. Actin filaments and the microtubules, the two most important parts of the cellular cytoskeleton were targeted so as to provide a model for the cellular response under unfavorable conditions since these two filamentous proteins play a pivotal role in the cell motility. Immunofluorescence assays were performed in which both the actin filaments and the microtubules were stained and viewed under the confocal microscope so as to quantify the results obtained from the biosensor. A wavelet transformation algorithm was applied to the results obtained from the biosensor so as to capture and compare the cellular fluctuations before and after the addition of the drug. The cytoskeletal filaments discussed in parts III and IV play a key role during the normal cellular processes and also determine the fate of the cell in hostile conditions. The way these filaments dynamically rearrange themselves under different conditions and the manner in which they offer resistance to forces and maintain their integrity holds the key to the cell's life. Monitoring

the structural changes of these filaments in response to opposing drugs and the corresponding effect of the cell's behavior thus provides a good model for determining the methods and materials behind each cellular process.

IV. Thesis Conclusions

The purpose of this master's level investigation was to showcase the usefulness and sensitivity of an inherently simple impedance biosensor setup in conducting *in vitro* cell based assays. This was shown by first demonstrating the ability of the biosensor to monitor the cellular activity over the surface of a gold electrode made viable for cell growth through a protein coat. A combination of quantification techniques were used to demonstrate the caliber of the biosensor to follow intracellular dynamics in response to specific drugs, which alter the key components of the cellular cytoskeleton. The importance of monitoring the change in intracellular mechanics in response to these drugs was evinced in the relevant chapters as to how it contributes to the field of drug discovery and tissue engineering. The final chapter was used as a means to assess the different possible ways to modify this biosensor setup to monitor the cellular behavior on artificial scaffolds, which holds great promise in tissue engineering. The methods used to quantify the results from the biosensor are very novel and give an accurate picture of the physical implications inside the cell. The confocal microscopy technique used to view the samples prepared by relevant immunofluorescence techniques stands out as a good quantification method and gave a good correlation coefficient with the obtained data. The wavelet analysis was applied to the acquired data so as to capture trends and events not visible to the naked eye. The wavelet transform gives an idea about the fluctuations in the data

which corresponds to the micromotion of the cells and enables us to compare the normal fluctuations to the same after external stimulation. Understanding the dynamics at the molecular level contributes immensely in drug discovery methods and therapeutic procedures for various pathologies. The combination of the biosensor setup along with the quantification procedures thus provides a good package to analyze cell cultures and perform cell based assays *in vitro*.

References

References

1. Cancer Quest Website. <http://www.cancerquest.org/index.cfm?page=162>
2. Borkholder, D.A., Cell Based Biosensors using microelectrodes, in Electrical Engineering. 1998, Stanford University.
3. Kay, N.e.a., 1954.
4. Baker, B.a.B., 1936; Schwan and Kay, 1956.
5. Barnett, L., 1987; Spence et al., 1979.
6. Thomas CA Jr, S.P., Loeb GE, Berwald-Netter Y and Okun CM, A miniature microelectrode array to monitor the bioelectric activity of cultured cells. *Experimental Cell Research*, 1972. 74: p. 61-66.
7. Novak, J.L., , and Wheeler, B. C, Recording from the Aplysia abdominal ganglion with a planar micro- electrode array. *IEEE Trans. on Biomedical Engineering BME*, 1986. 33(2): p. 196-202.
8. Jimbo, Y., and Kawana, A. Electrical stimulation of cultured neural cells by planar electrode array. in *Annual International Conference of the IEEE Engineering in Medicine and Biology Society*. 1990. Tokyo, Japan.
9. O'Shea, P., Goodwin, B., and Ridge, I, A vibrating electrode anslysis of extracellular ion currents in *Acetabularia acetabulum*. *Journal of Cell Science*, 1990. 97: p. 503-508.
10. Zarowitz BJ, P.A., Bioelectrical impedance in clinical practice. 1996, Department of Pharmacy Services, Henry Ford Hospital, Wayne State University: Detroit.
11. Schloerb PR, F.J., Delcore R, Kindscher JD, Bioelectrical impedance in the clinical evaluation of liver disease. 1996.
12. Bucciante, A.P.B.R.L.P.G., Body Fluid Overload and Bioelectrical Impedance Analysis in Renal Patients. 1996.
13. Joachim WEGENER, C.R.K., and Ivar GIAEVER, BIOPHYSICAL SENSORS AND ACTUATORS TO STUDY ANIMAL CELLS IN SITU.
14. Giaever, I.a.K., C.R., A morphological biosensor for mammalian cells. *Nature*, 1993. 366: p. 591-592.
15. Keese, C.R.a.G., I., A Whole Cell Biosensor Based on Cell-Substrate Interactions. *IEEE Proc. Biomed. Eng*, 1990. 12: p. 500-501.
16. Lo, C.-M., C.R. Keese, and I. Giaever, Impedance Analysis of MDCK Cells Measured by Electric Cell-Substrate Impedance Sensing. *Biophys. J.*, 1995. 69: p. 2800-2807.
17. Giaever, I. and C.R. Keese, Micromotion of Mammalian Cells Measured Electrically. *Proc. Natl. Acad. Sci. USA*, 1991. 88: p. 7896-7900.
18. Xiao, C., et al., Assessment of Cytotoxicity Using Electric Cell-Substrate Impedance Sensing: Concentration and Time Response Function Approach. *Anal. Chem.*, 2002. 74: p. 5748-5753.
19. Lo, C.M., Ferrier, J., Impedance analysis of fibroblastic cell layers measured by electric cell-substrate impedance sensing. *Physical Review*, 1998. 57(6): p. 6982-6987.

20. Charboneau, A.L., Singh, V., Yu, T.X., Newsham, I.F., Suppression of growth and increased cellular attachment after expression of DAL-1 in MCF-7 breast cancer cells. *International Journal of Cancer*, 2002. 100(2).
21. Xiao, C., Luong, J., On-Line Monitoring of Cell Growth and Cytotoxicity Using Electric cell-substrate Impedance Sensing (ECIS). *Biotechnol Prog.* 19: p. 1000-1005.
22. Luong, J.H.T., Habibi-Rezaei, M., Meghrou, J., Xiao, C., Male, K.B., Kamen, A., Monitoring motility, spreading, and mortality of adherent insect cells using an impedance sensor. *Analytical Chemistry*, 2001. 73(8): p. 1844-1848.
23. Sriram, P.S., Mohammed, K.A., Nasreen, N., Hardwick, J., Van Horn, R., Sanders, K., Antony, V.B., Adherence of ovarian cancer cells induces pleural mesothelial cell (PMC) permeability. *Oncology Research*, 2001. 13(2): p. 79-85.
24. Keese, C.R., Wegener, Joachim, Walker, Sarah R., and Giaever, Ivar., Electrical Wound-healing assay for cell in vitro. *PNAS*, 2004. 101: p. 1554-1559.
25. Moy, A.B., et al., Histamine and Thrombin Modulate Endothelial Focal Adhesion through Centripetal and Centrifugal Forces. *J. Clin. Invest.*, 1996. 97(4): p. 1020-1027.
26. Sawhney, R.S., Sharma, B., Humphrey, L.E., Brattain, M.G., Integrin $\alpha 2$ and extracellular signal-regulated kinase are functionally linked in highly malignant autocrine transforming growth factor- α -driven colon cancer cells. *J. Biological Chemistry*, 2003. 278(22): p. 19861-19869.
27. Keese, C.R., et al., Real-Time Impedance Assay to Follow the Invasive Activities of Metastatic Cells in Culture. *Biotechniques*, 2002. 33: p. 842-850.
28. Wegener, J., Keese, C.R., Giaever, Recovery of Adherent Cells After In Situ Electroporation Monitored Electronically. *BioTechniques*, 2002. 33(2).
29. Xiao, C., et al., An In-Depth Analysis of Electric Cell-Substrate Impedance Sensing To Study the Attachment and Spreading of Mammalian Cells. *Anal. Chem.*, 2002. 74: p. 1333-1339.
30. Benjamin, C., QUANTITATIVE ENDOTHELIAL CELL MONOLAYER IMPEDANCE SENSING AND ANALYSIS, in *Mechanical, Aerospace and Biomedical Engineering*. 2003, University of Tennessee: Knoxville.
31. De Blasio, B.F., Laane, M., Walmann, T., and Giaever, I, Combining optical and electrical impedance techniques for quantitative measurement of confluence in MDCK-I cell cultures. *BioTechniques*, 2004. 36: p. 650-662.
32. Stebbings, H., Cytoskeleton-dependent transport and localization of mRNA. *Int. Rev. Cytol*, 2001.
33. Bertorello AM, K.Y., Smith K, Leibiger IB, Efendiev R, Pedemonte CH, Borisov G, Sznajder JJ, Analysis of Na^+ , K^+ -ATPase Motion and Incorporation into the Plasma Membrane in Response to G Protein-coupled Receptor Signals in Living Cells. *Mol. Biol. Cell*, 2003. 14(3): p. 1149-1157.
34. Vorobjev IA, A.I., Grigoriev IS, Borisov GG., Microtubule dynamics in living cells: direct analysis in the internal cytoplasm. *Cell Biology International*, 2003. 27(3): p. 294-297.
35. Pollard TD, B.G., Cellular motility driven by assembly and disassembly of actin filaments. *Review. Erratum in: Cell*, 2003. 112(4): p. 453-465.

36. I.A. Vorobjev, I.S.G., G.G. Borisov, Ontogenesis, Microtubule dynamics in cultured cells. Russian, 2000. 31(6): p. 420-428.
37. Svitkina, T.M.a.B., G.G., Correlative Light and Electron Microscopy of the Cytoskeleton of Cultured Cells. *Methods in Enzymology*, 1998. 298: p. 570-92.
38. Tatyana M. Svitkina, a.G.G.B.J.C.S., Cytoplasmic assembly of microtubules in cultured cells. *J. Cell Sci.*, 1997. 110: p. 2635-2645.
39. Svitkina, T.M., Verkhovsky, A. B., Improved Procedures For Electron Microscopic Visualization Of The Cytoskeleton Of Cultured Cells. *Journal of Structural Biology*, 1995. 115(290-303).
40. Kevin A. Edwards, M.D., Ruth A. Montague, Nate Weymouth, and Daniel P. Kiehart, GFP-Moesin Illuminates Actin Cytoskeleton Dynamics in Living Tissue and Demonstrates Cell Shape Changes during Morphogenesis in *Drosophila*.
41. V Jayme L. Martys, C.-L.H., * Ronald K. H. Liem, and Gregg G, Intermediate Filaments in Motion: Observations of Intermediate Filaments in Cells Using Green Fluorescent Protein-Vimentin.
42. Waterman-Storer, V.C.M., Microtubules and Microscopes: How the Development of Light Microscopic Imaging Technologies Has Contributed to Discoveries about Microtubule Dynamics in Living Cells. 1998.
43. P. Vallotton, A.P., * C. M. Waterman-Storer, y E. D. Salmon, z and G. Danuser, Recovery, Visualization, and Analysis of Actin and Tubulin Polymer Flow in Live Cells: A Fluorescent Speckle Microscopy Study.
44. Kylie A. Hotchkiss, A.W.A., R.G.R. Radma Mahmood, and a.E.L.S. Joseph A. Sparano,. Inhibition of Endothelial Cell Function in Vitro and Angiogenesis in Vivo by Docetaxel (Taxotere): Association with Impaired Repositioning of the Microtubule Organizing Center. *Molecular Cancer Therapeutics*, 2002. 1: p. 1191-1200.
45. ALEXANDER D. VERIN, A.B., PEIYI WANG, FENG LIU, PATRICE BECKER, KONSTANTIN BIRUKOV, AND JOE G. N. GARCIA, Microtubule disassembly increases endothelial cell barrier dysfunction: role of MLC phosphorylation. 2000.
46. Grinnell, F., *Int. Rev. Cytol*, 1978. 53.
47. FB, S., Actin. In: *Studies from the Institute of Medical Chemistry University Szeged*. 1942. 2: p. 3-15.
48. J.-VICTOR SMALL, K.R., 1 PENELOPE HAHNE, 1 AND KURT I. ANDERSON², Visualising the Actin Cytoskeleton.
49. Paul A. Janmey¹, J.X.T.a.C.F.S., Actin Filaments, in *BioPhysics Text Book Online*.
50. Céline Revenu, R.A., Sylvie Robine and Daniel Louvard, THE CO-WORKERS OF ACTIN FILAMENTS: FROM CELL STRUCTURES TO SIGNALS.
51. A, H., *Confocal Microscopy for Biologists: An Intensive Introductory Course*. 2000. p. 2-9.
52. Michalet X, e.a., The Power and Prospects of Fluorescence Microscopies and Spectroscopies. *Annu Rev Biophys Biomol Struct*, 2003. 32: p. 161-182.
53. Stevens JK, e.a., *Three- Dimensional Confocal Microscopy: Volume Investigation of Biological Systems*, L.A. Press, Editor.

54. Addison, P.S., Illustrated Wavelet Transform.
55. Polikar, R., A wavelet tutorial:
engineering.rowan.edu/~polikar/WAVELETS/WTtutorial.html.
56. Tilney et al 1973.
57. Albertini, D.F., Stage-specific induction of microtubule organizing centers by taxol during meiotic maturation of rat oocytes. J Cell Biol, 1981.

Appendices

Appendix I: Figures

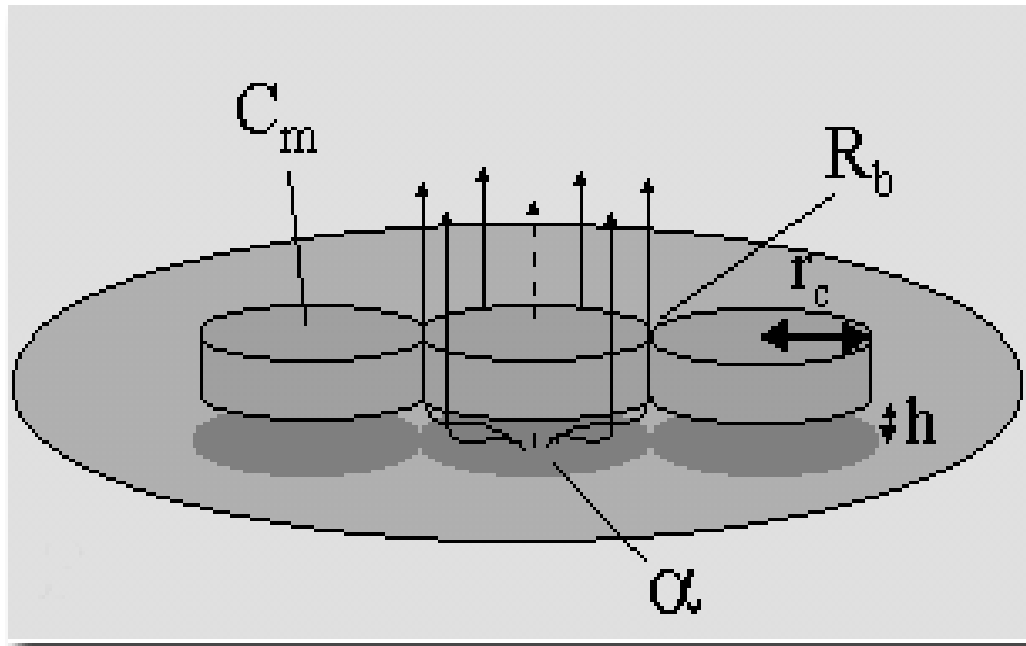
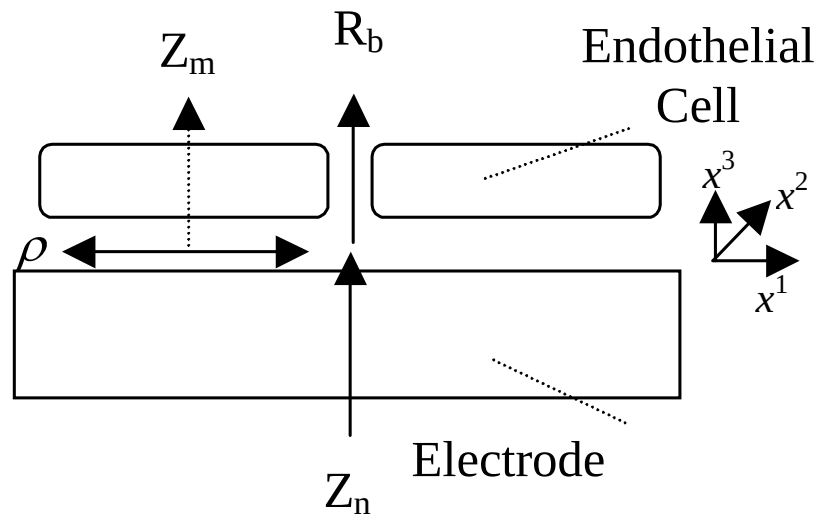
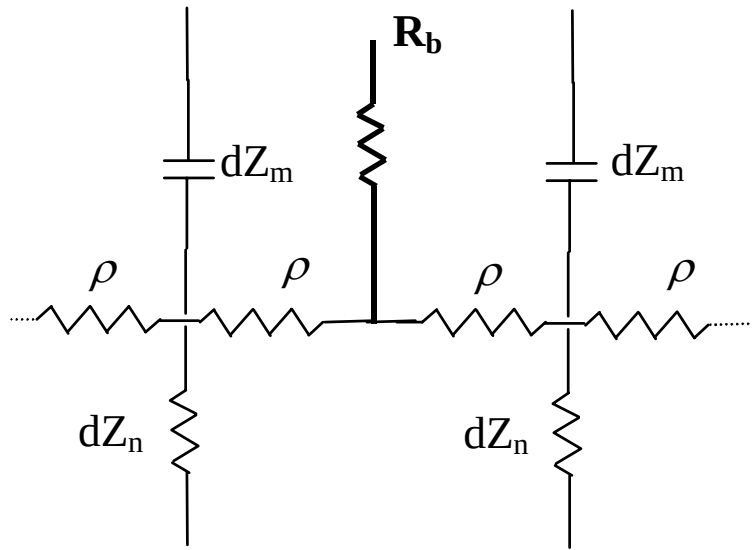


Figure A.1.1: Cells modeled as round disk shaped objects and the corresponding resistances R_b , α and the capacitance across the cell membrane are represented. The current flow through the cell-electrode interface depends on these three parameters.



(a)



(b)

Figure A.1.2: The path of current flow through the endothelial cell-substratum boundary (a) and the equivalent electrical circuit (b) along with the impedances, current density and voltage drops at different junctions.

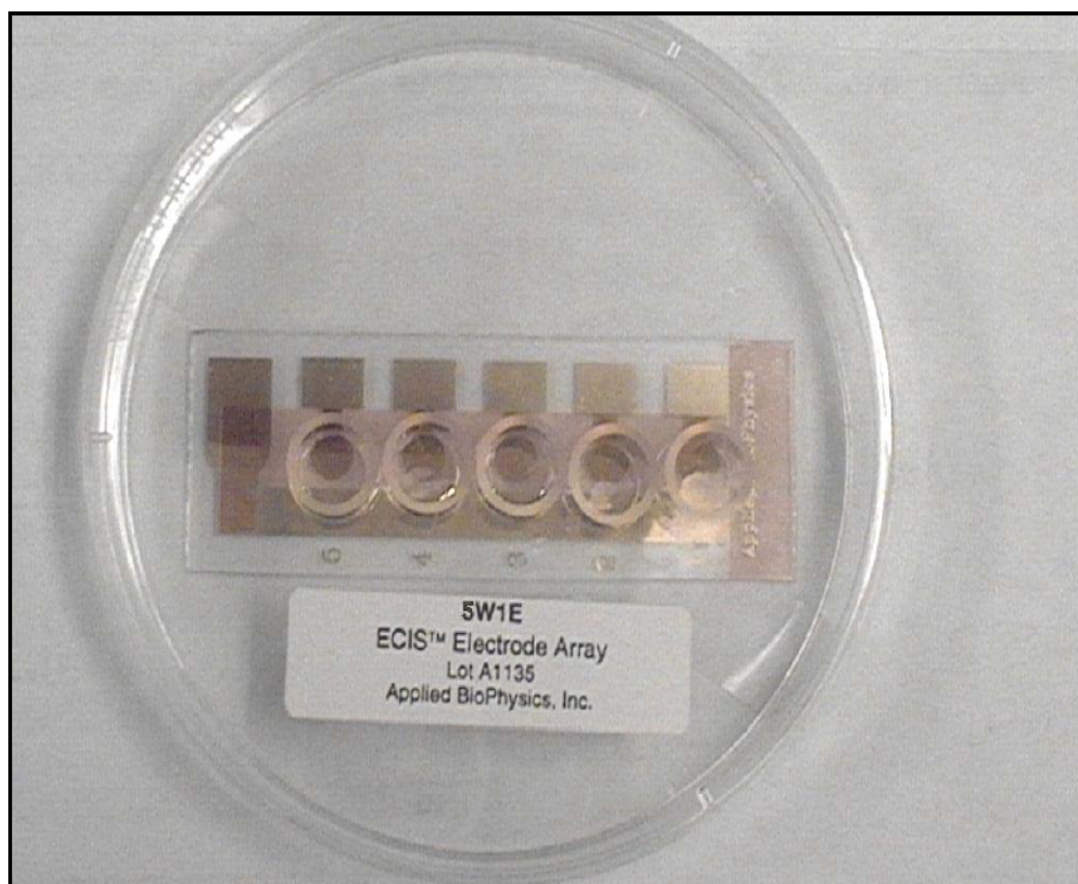


Figure A.1.3: The Base electrode which forms the cardinal part of the biosensor setup along with the series of five gold microelectrodes housed inside the plastic wells

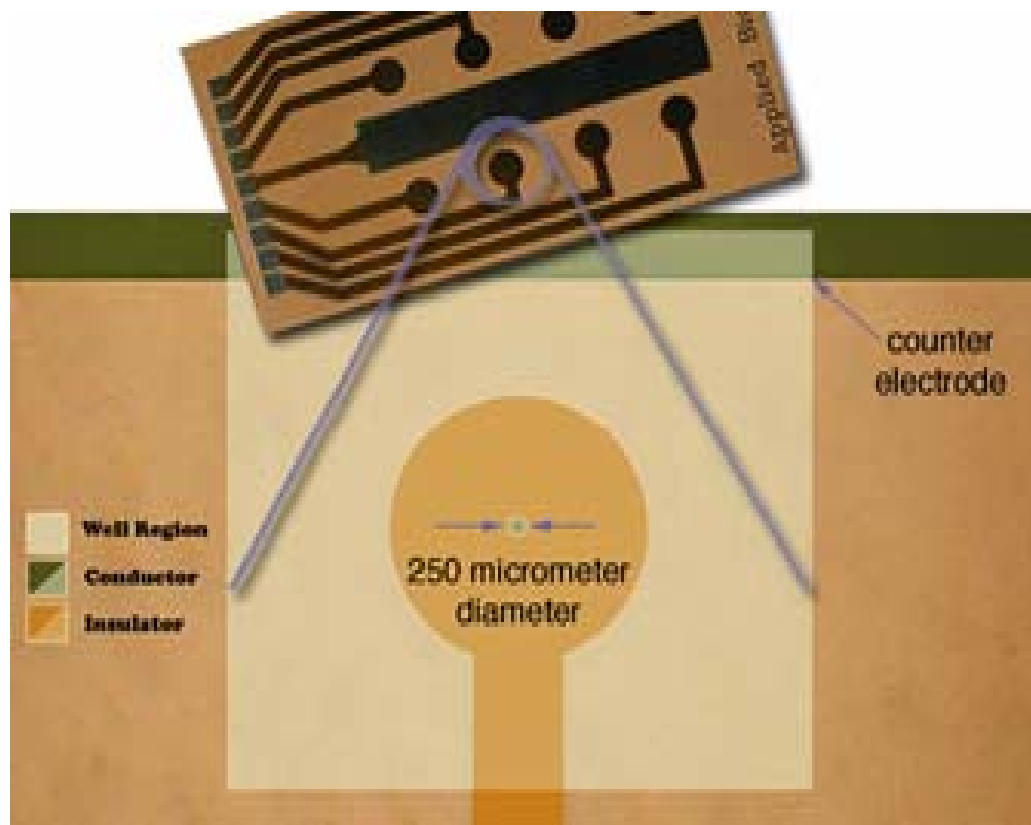


Figure A.1.4: The structure and surface properties of the microelectrode in conjunction with the counter electrode is represented

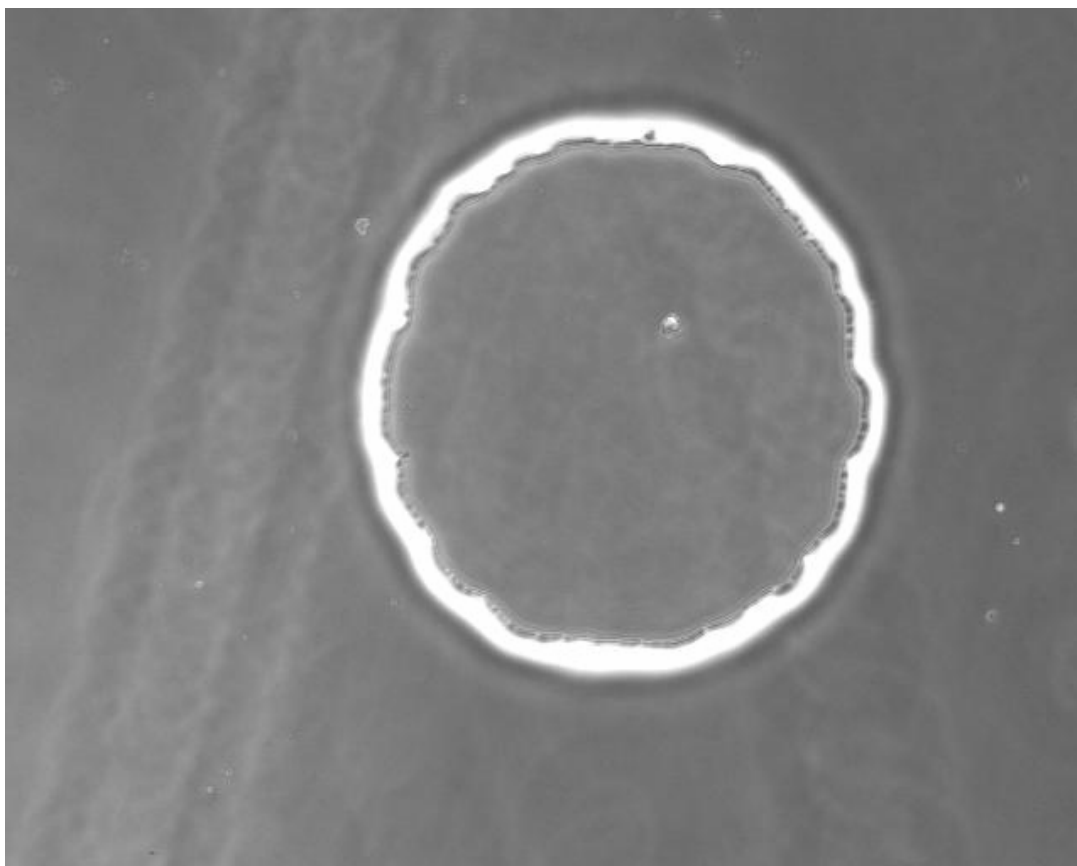


Figure A.1.5: Micrograph of the gold measuring electrode with no cells on it

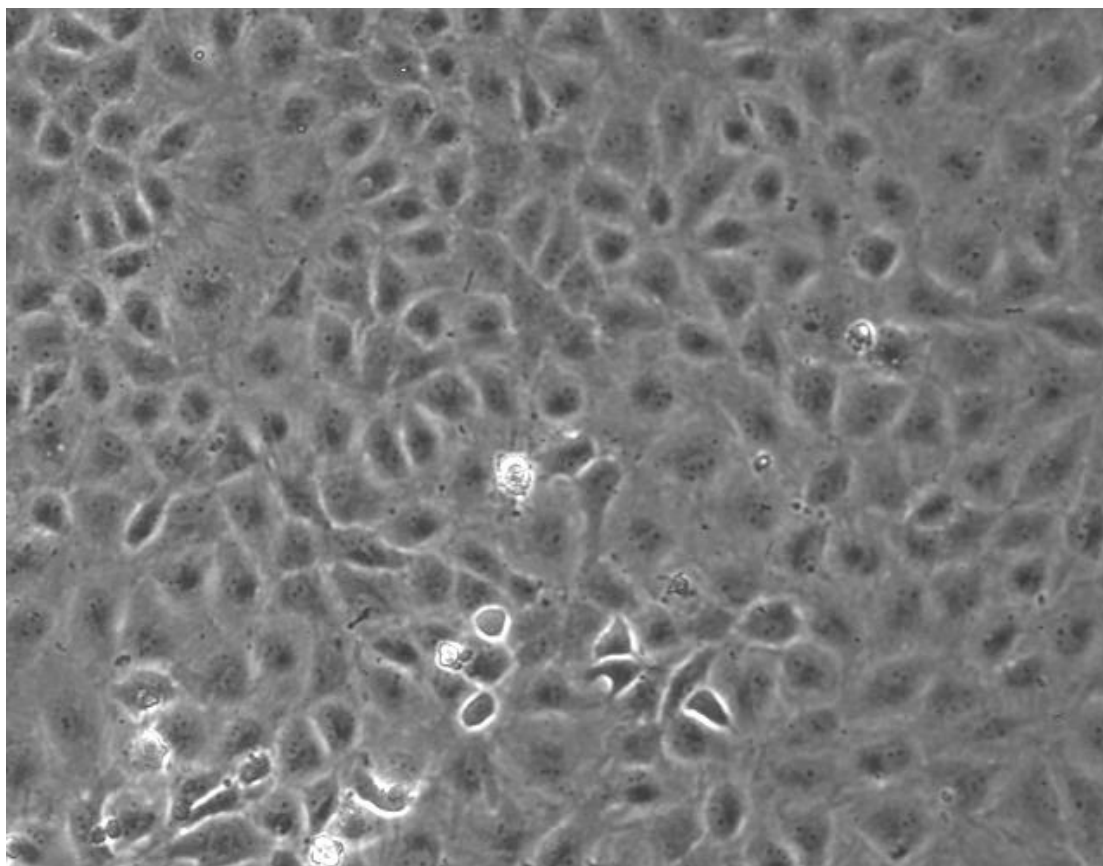
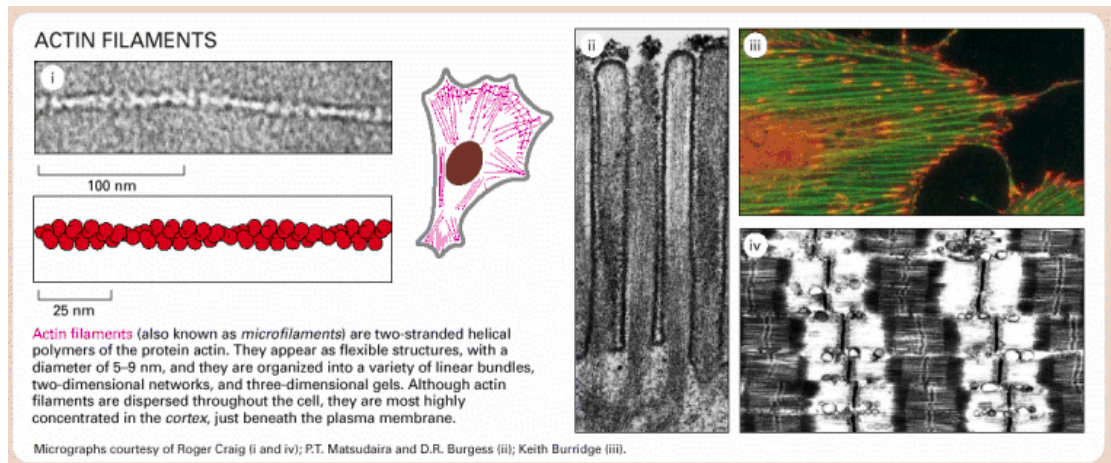
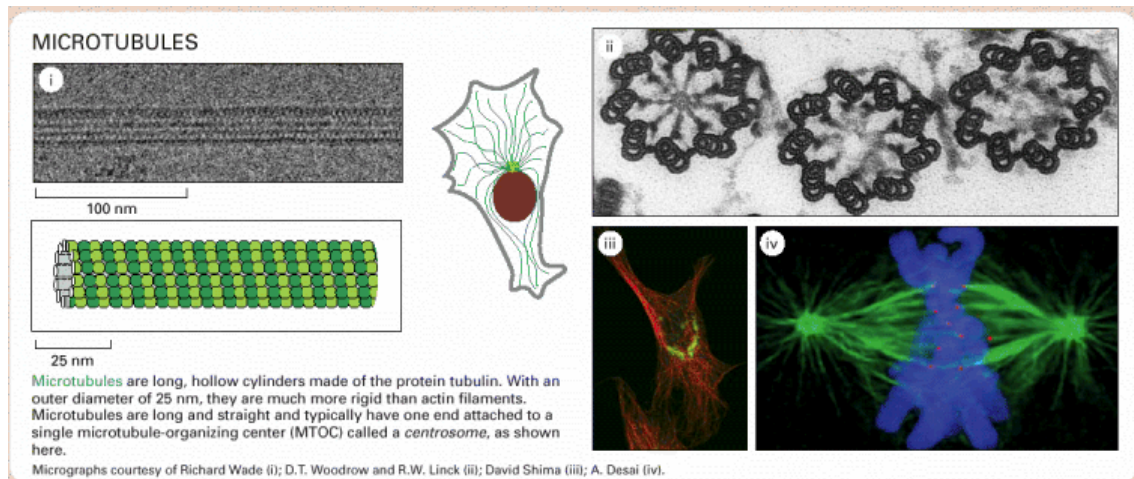


Figure A.1.6: Micrograph of the cultured endothelial cells and the Cytoskeleton structure. (a)The cobblestone morphology can be observed and this figure is taken of endothelial cells growing over a petri dish that is pre-treated for cell growth



(b)



(c)

Figure A.1.6 Continued. (b) The actin filaments of the cellular cytoskeleton which play a pivotal role in providing mechanical strength and aids in cellular locomotion. (c) The microtubules which performs an important function in mitosis and a key role in intracellular transport.

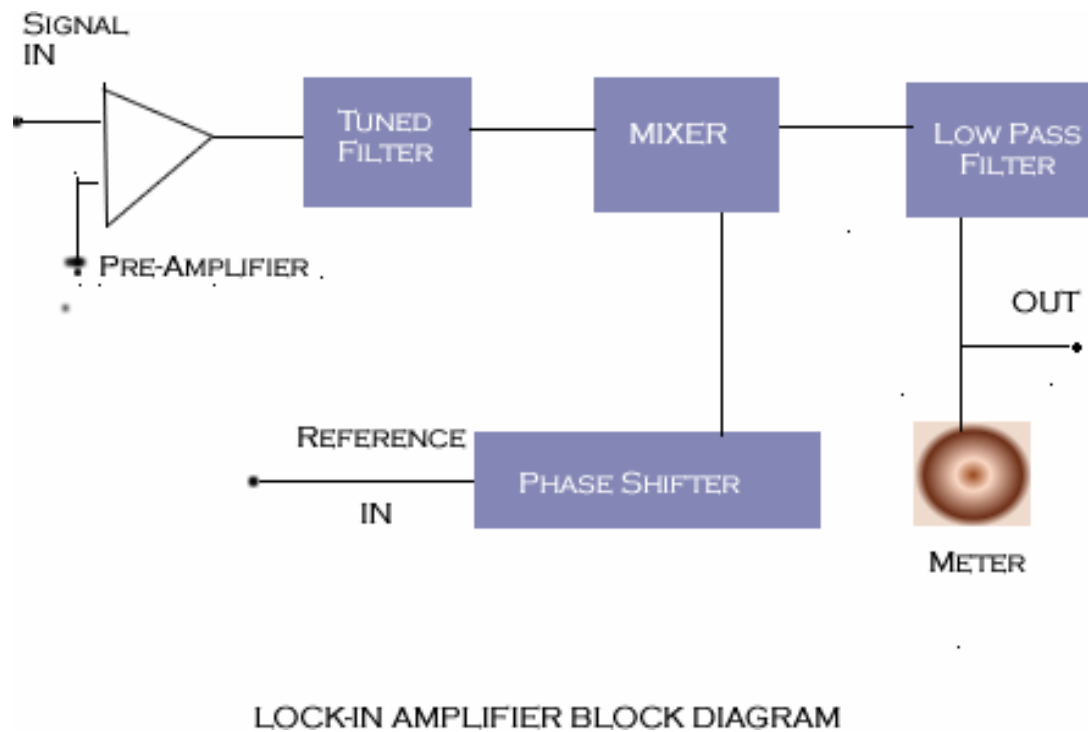


Figure A.1.7: A typical block diagram of a Lock-in Amplifier is represented. The lock-in amplifier is used here to stimulate the cells using a non-invasive current of 1 μA and also phase-sensitively detect the cellular response

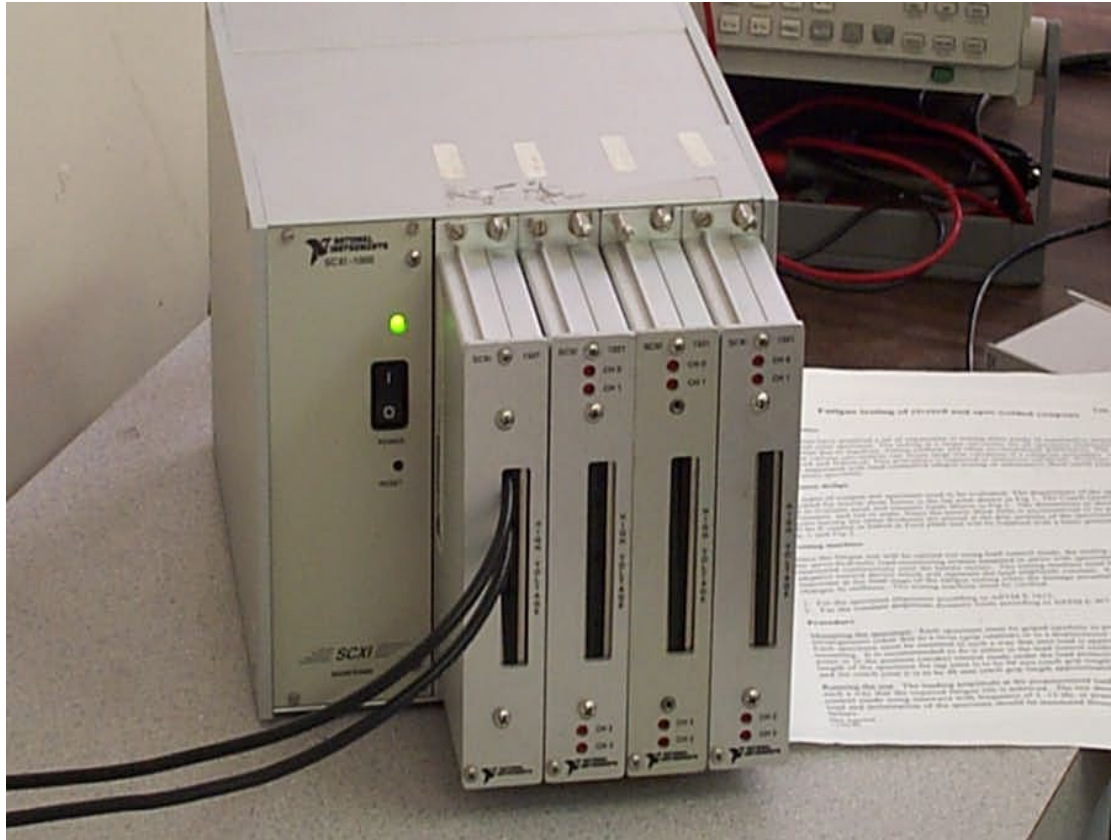


Figure A.1.8: The National Instruments DAQ system is shown here. We used the SCXI 1000 series which collected the data from the biosensor through the lock-in amplifier according to instructions set by the Virtual Instrumentation program coded in LabVIEW

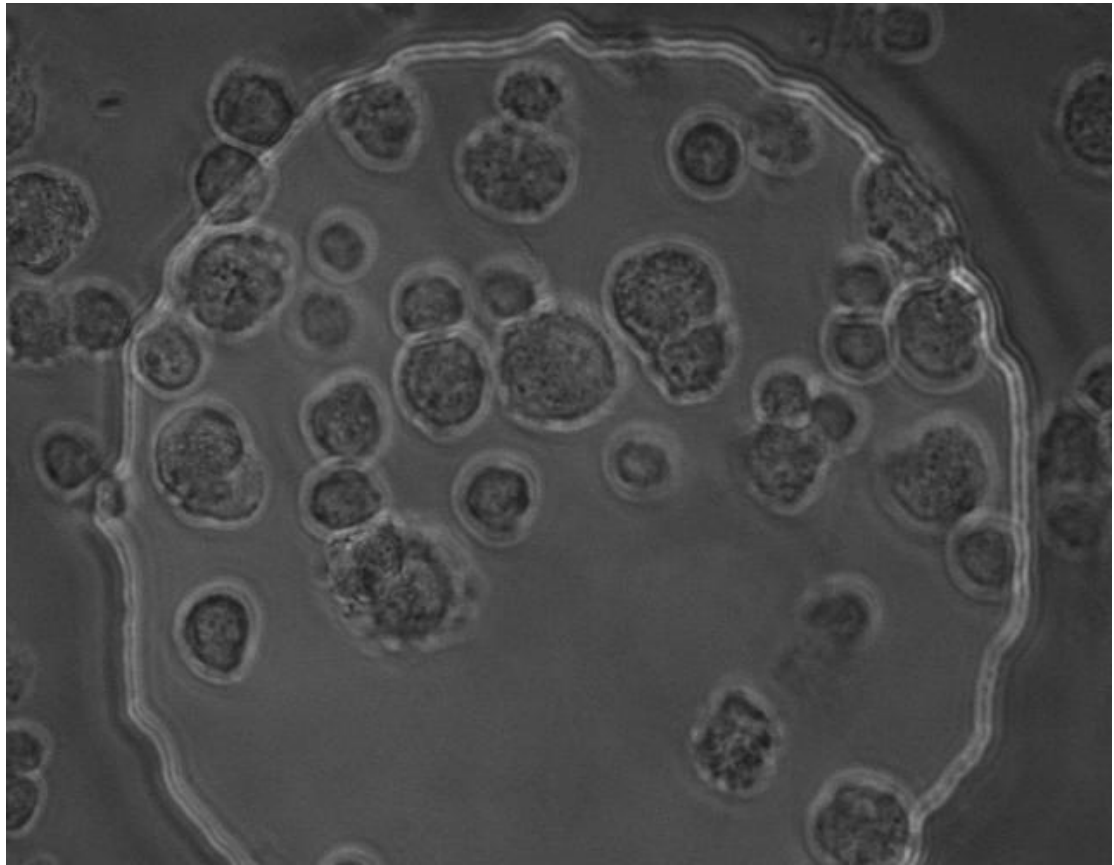


Figure A.1.9: Micrograph showing the endothelial cell adhesion on top of the measuring electrode. It has to be noted that this formation of a monolayer on top of the microelectrode is the underlining principle of this whole biosensor setup and provides a non-invasive approach to monitor cells *in vitro*

The Cell Impedance Biosensor Setup

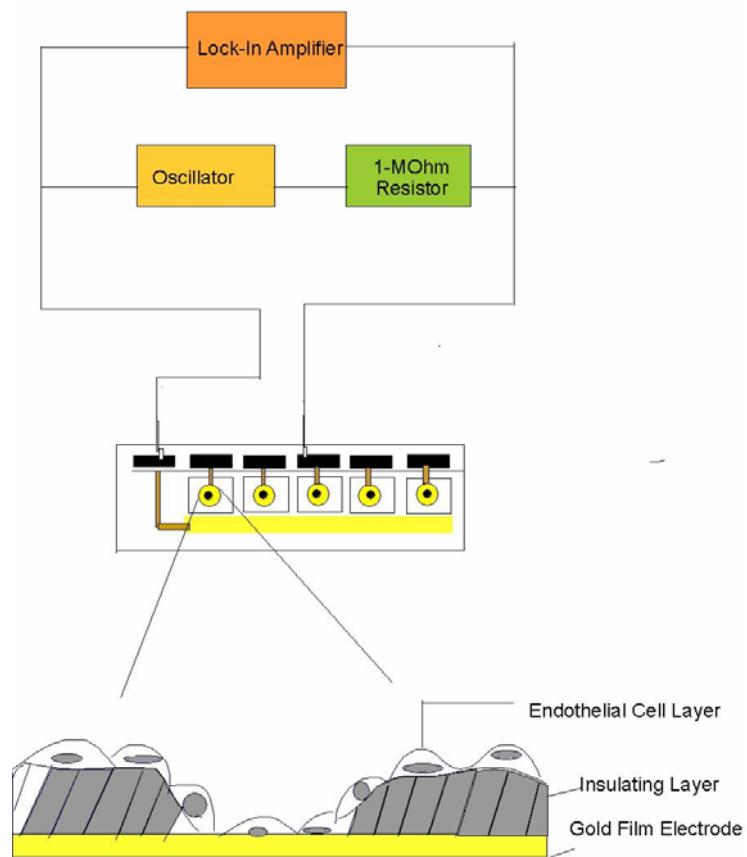


Figure A.1.10: The Cellular Impedance Biosensor Setup. (a) A pictorial representation of the cellular impedance biosensor setup used to monitor cell cultures in real-time.

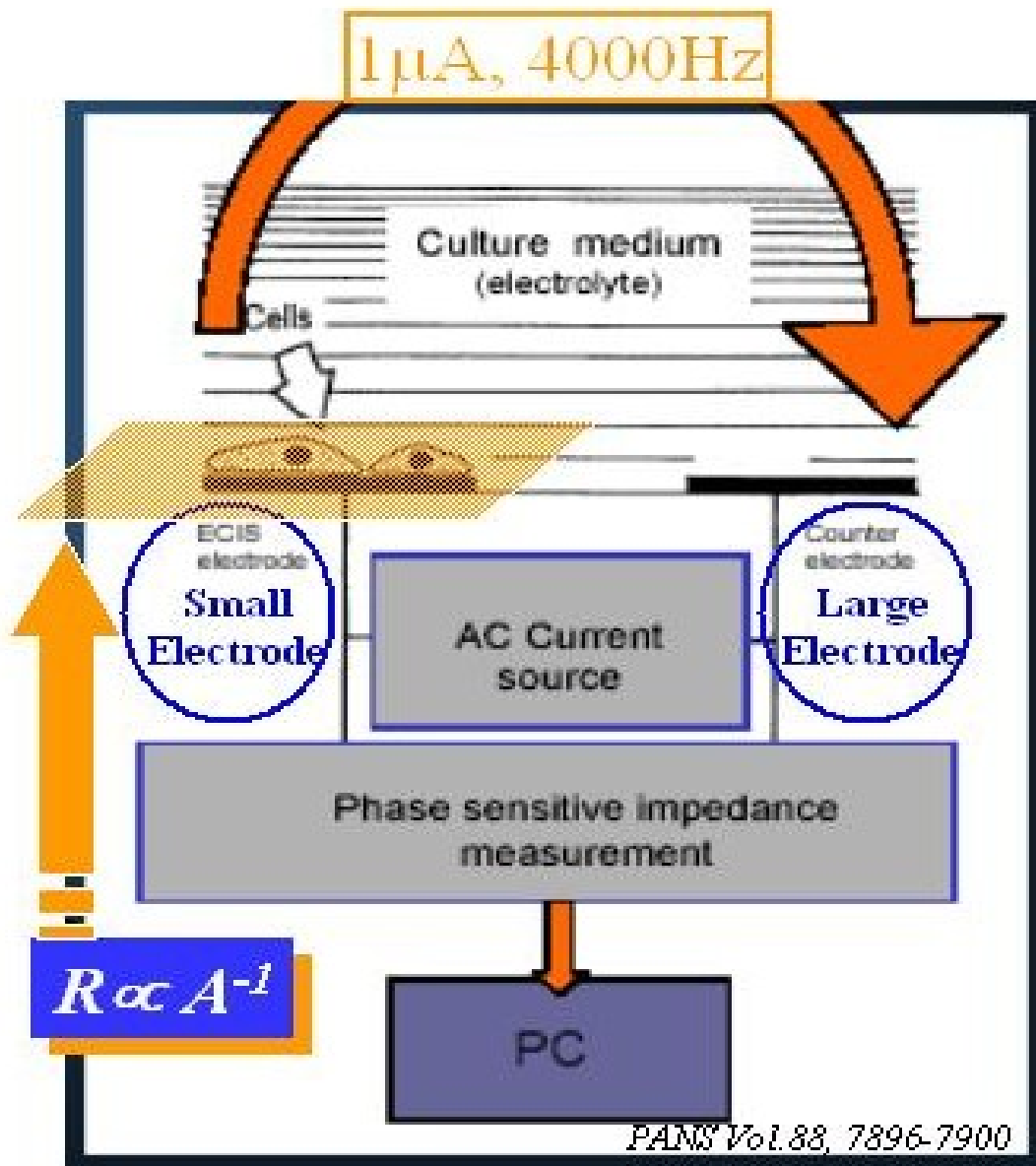


Figure A.1.10 Continued. (b): The principle of operation of the biosensor is represented here. The direction of current flow from the microelectrode to the counter electrode is evinced here and the general instrumentation used are also represented in the figure

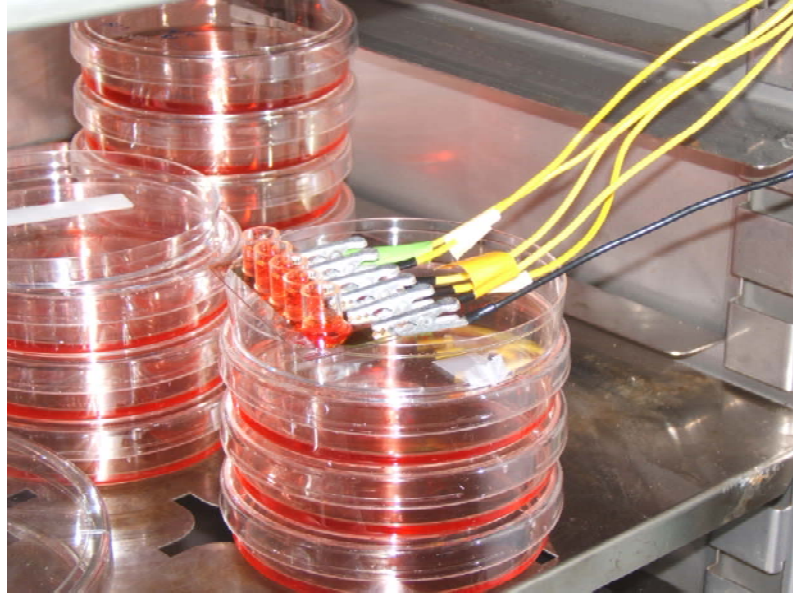
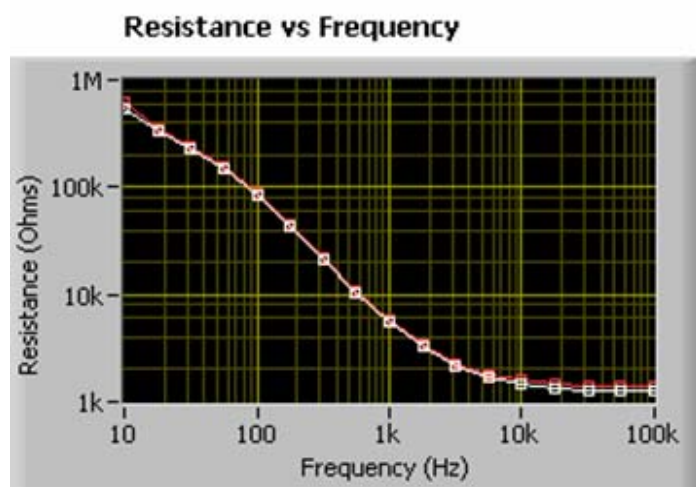
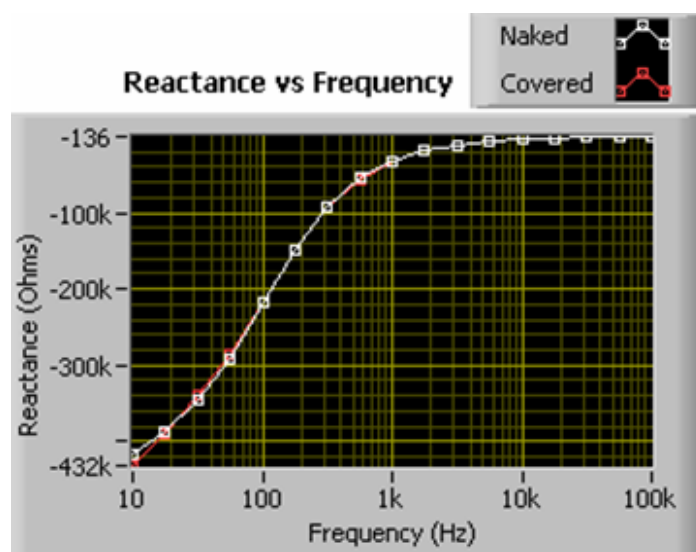


Figure A.1.11: A digital picture representing the whole instrumentation setup in the lab that we used for our study



(a)



(b)

Figure A.1.12: The graph shows the naked and cell covered resistance and reactance as a function of frequency for the initial scan

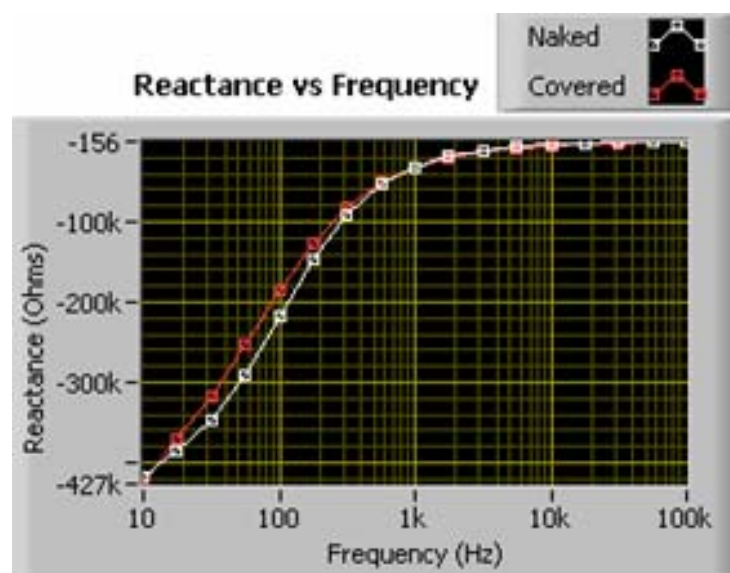
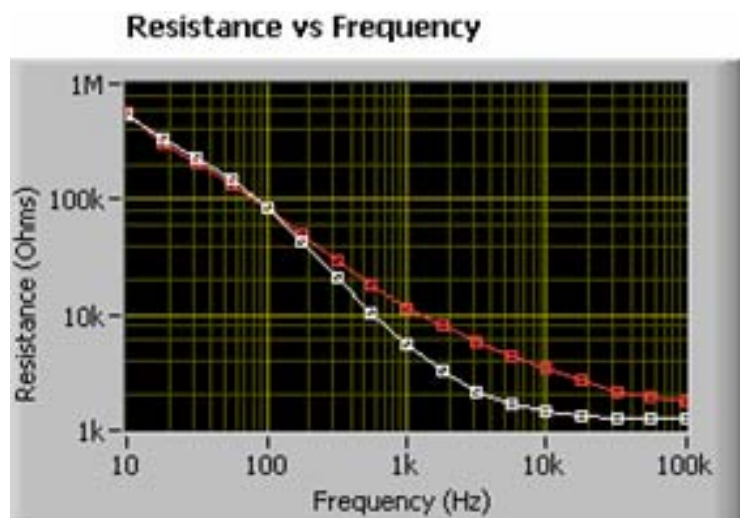


Figure A.1.13: The graph shows the naked and cell covered resistance and capacitance as a function of frequency after 25 scans. The difference between the cell covered and naked electrode scans can be easily seen between the 1-10 KHz range.

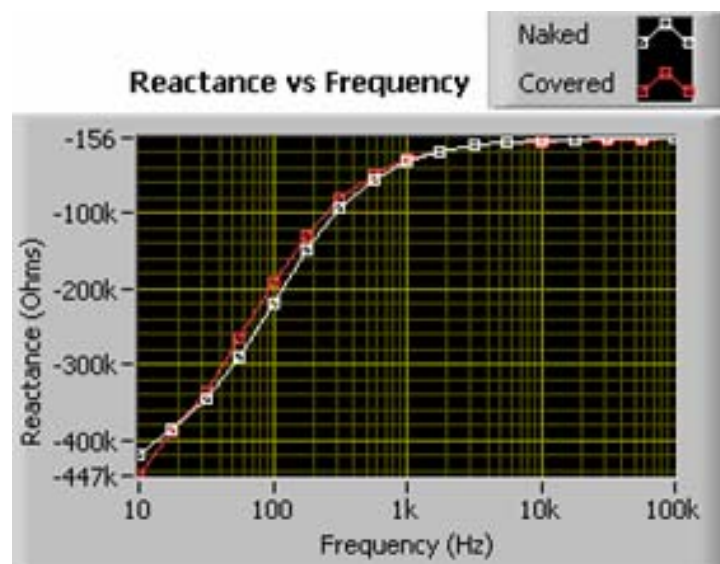
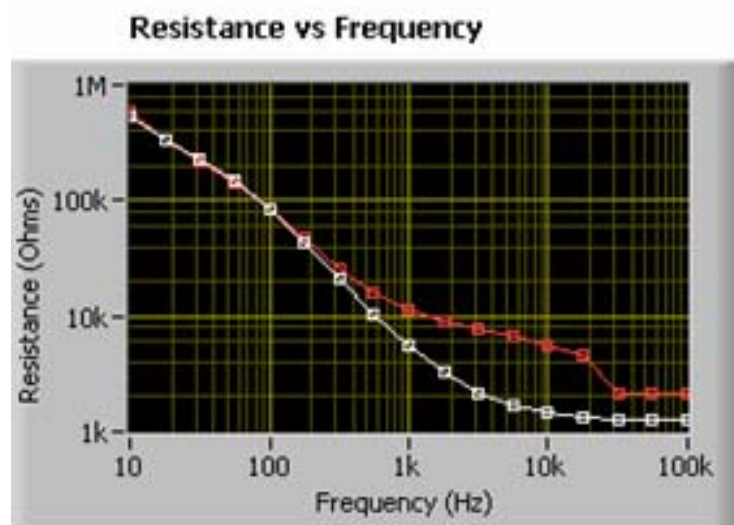


Figure A.1.14: The graph shows the naked and cell covered resistance and capacitance as a function of frequency after 50 scans. The difference between the cell covered and naked electrode scans is more pronounced between the 1-10 KHz range

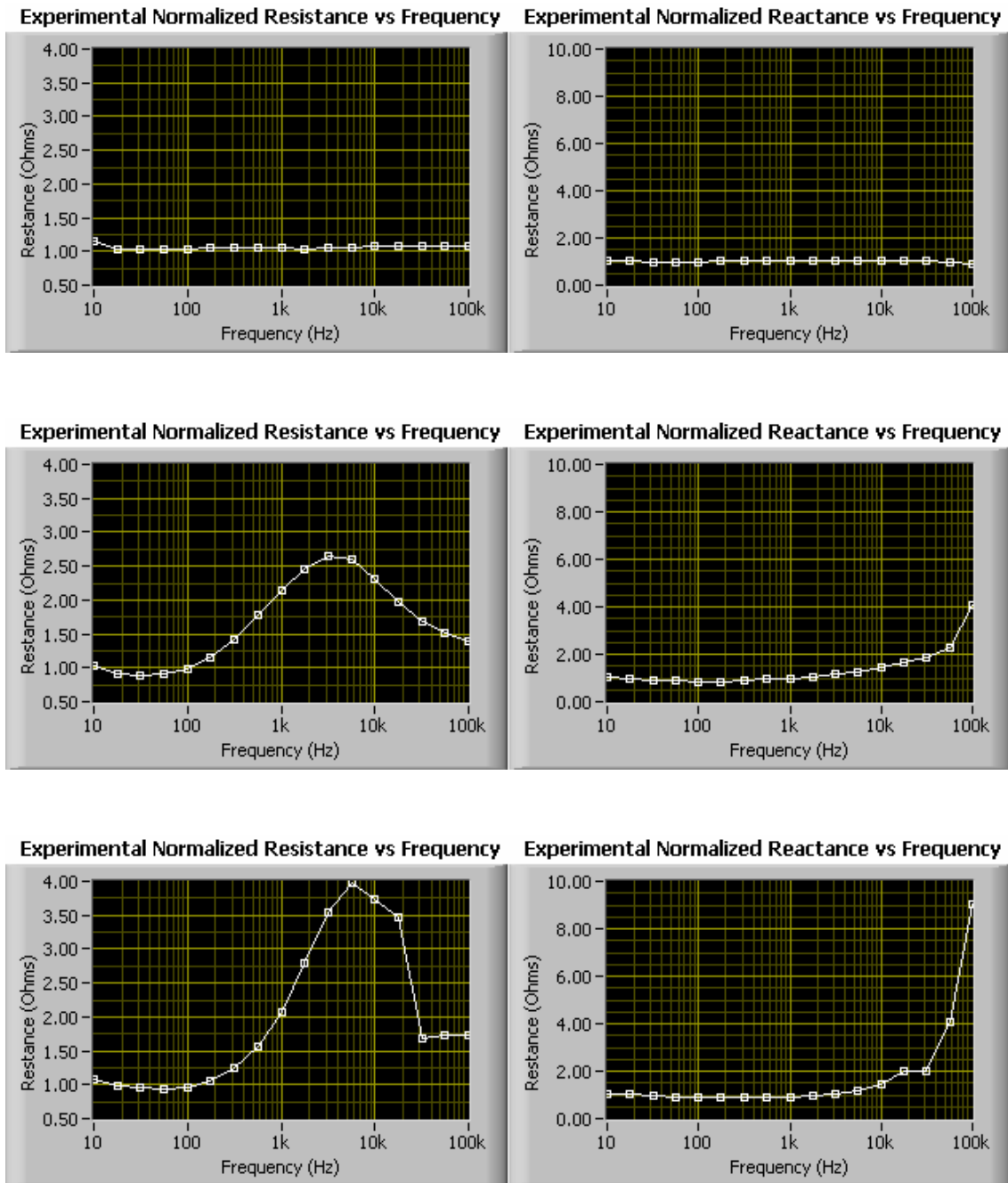
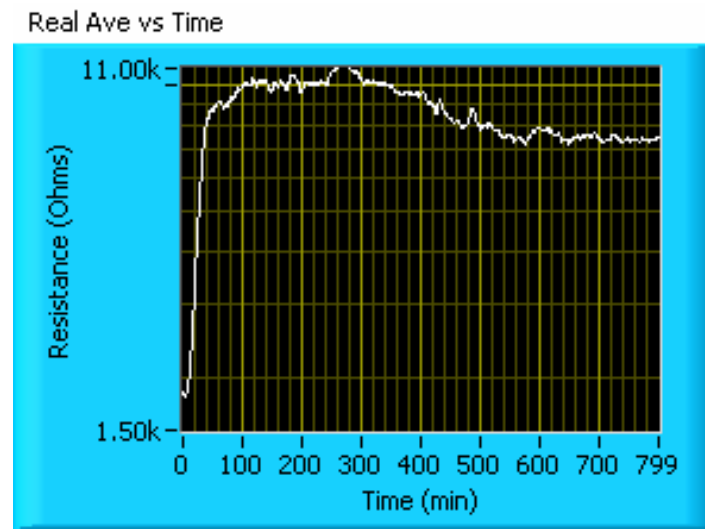
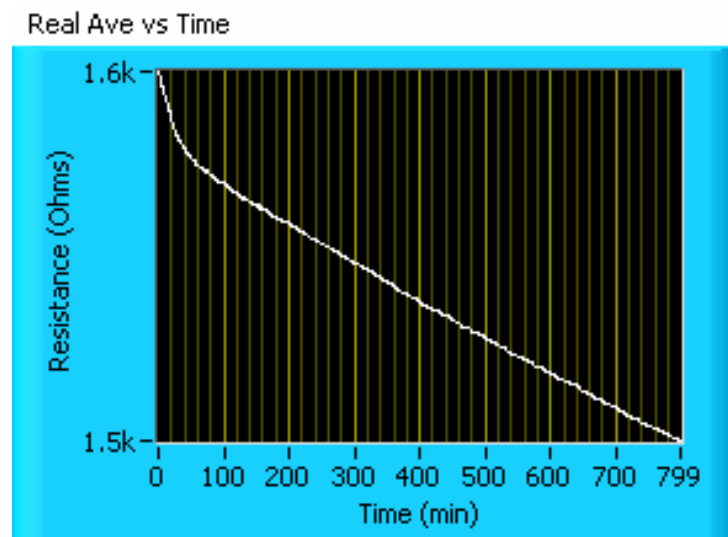


Figure A 1.15: A series of normalized impedances as a function of frequency elucidating the effect of formation of the monolayer on top of the electrode. It can be seen that after a complete attach the impedance measured can be as high as 6-8 times the naked electrode impedance



(A)



(B)

Figure A.1.16: A time series of the naked and cell covered electrode scans. The fluctuations in the acquired data should be noted here. These fluctuations correspond to the cellular micromotion on top of the microelectrode after formation of a confluent layer

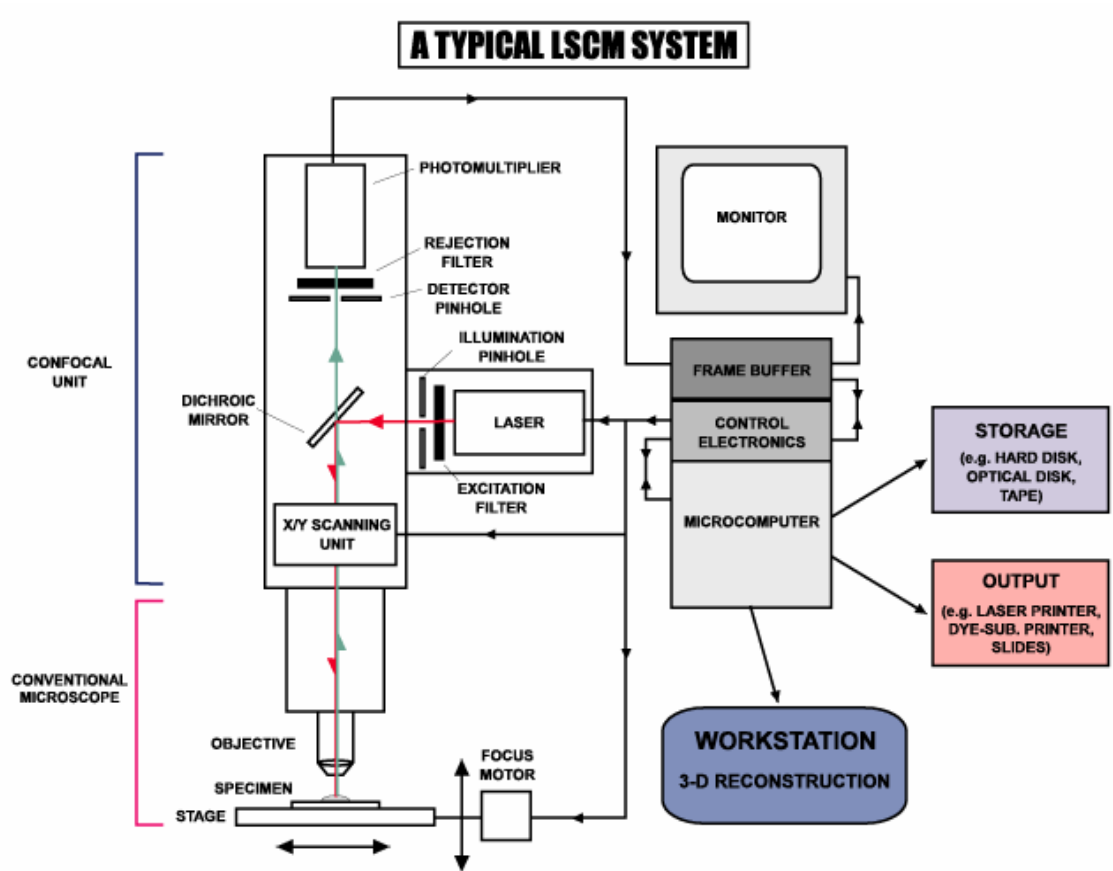


Figure A.1.17: The figure represents the layout of a typical confocal microscope. The special feature of the confocal microscope is its ability in extracting optical slices of samples that is not possible with a traditional microscope

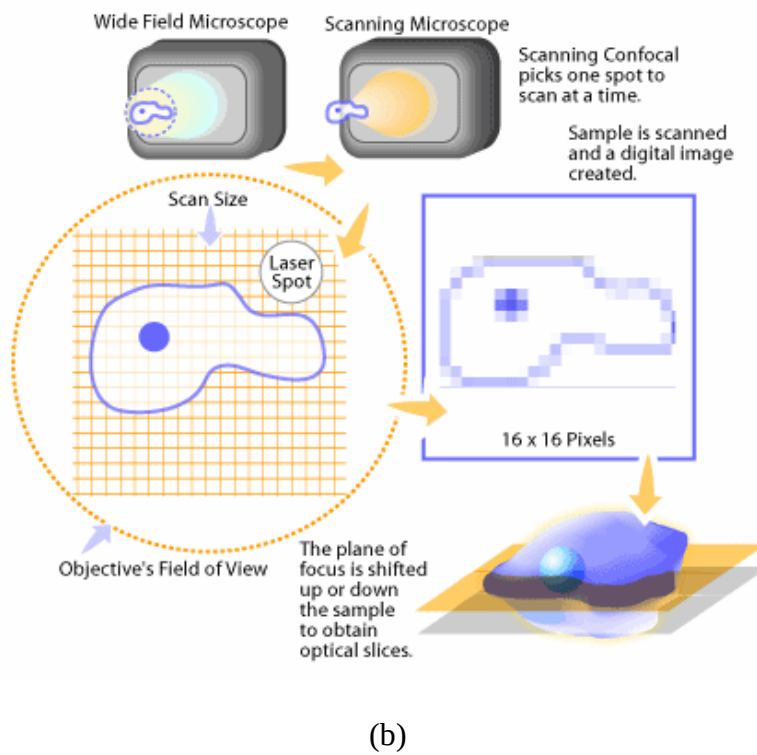
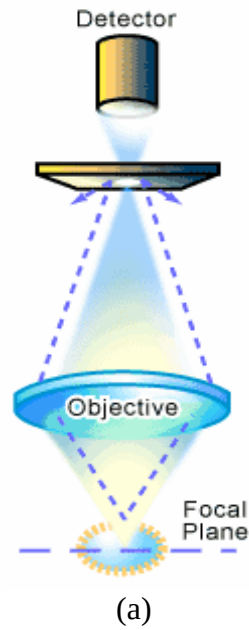
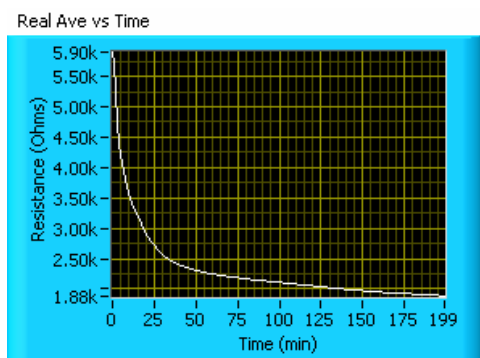
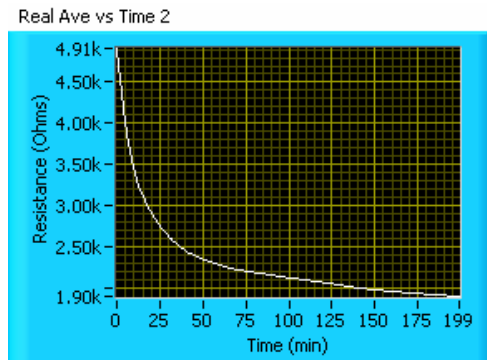


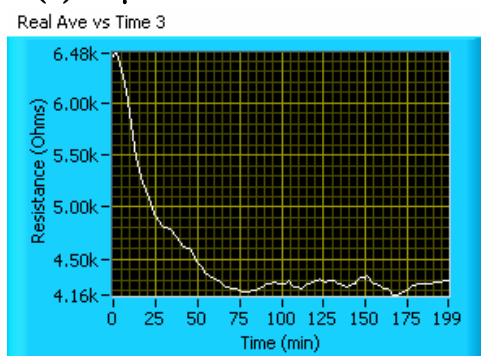
Figure A.1.18: The principle behind the confocal microscope in obtaining 3-D representation of the sample by collecting slices across different sections of the sample



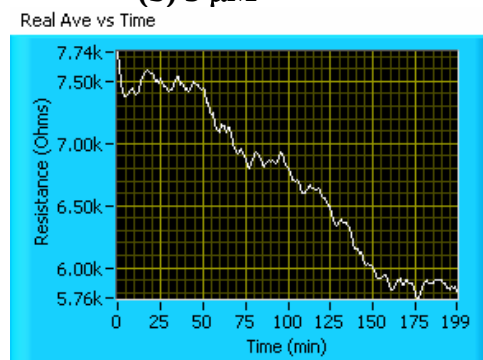
(a) 30 μ M



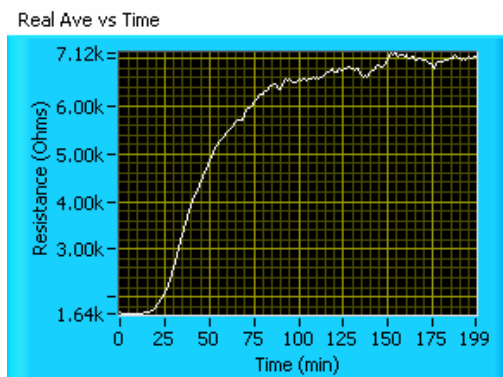
(b) 3 μ M



(c) 0.3 μ M



(d) 0.03 μ M



(e) Attach scan

Figure A.1.20: The figure represents a series of data obtained after addition of Cytochalasin D in concentrations ranging from 30uM to .003uM

Intitial Study with Widely Varying Concentrations of Cytochalasin

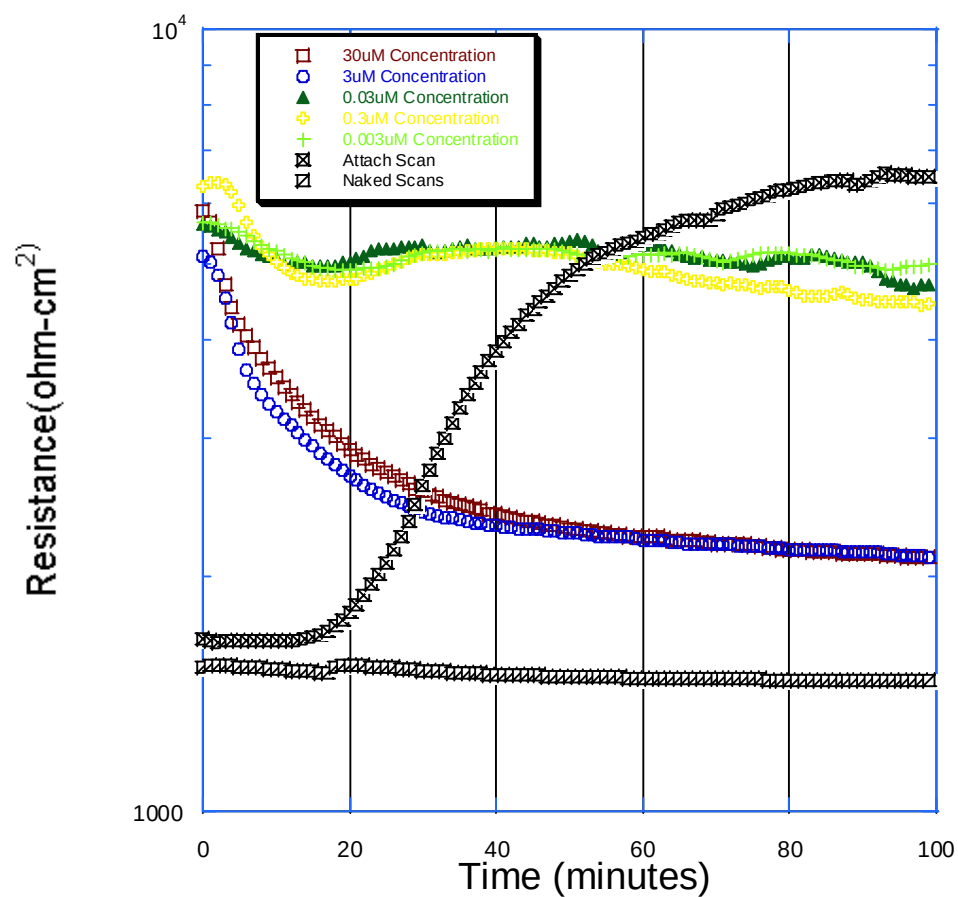


Figure A1.1.21: As we can see from the results the data we obtained was reflective of cellular behavior but with a irregular pattern between 3uM and .3uM

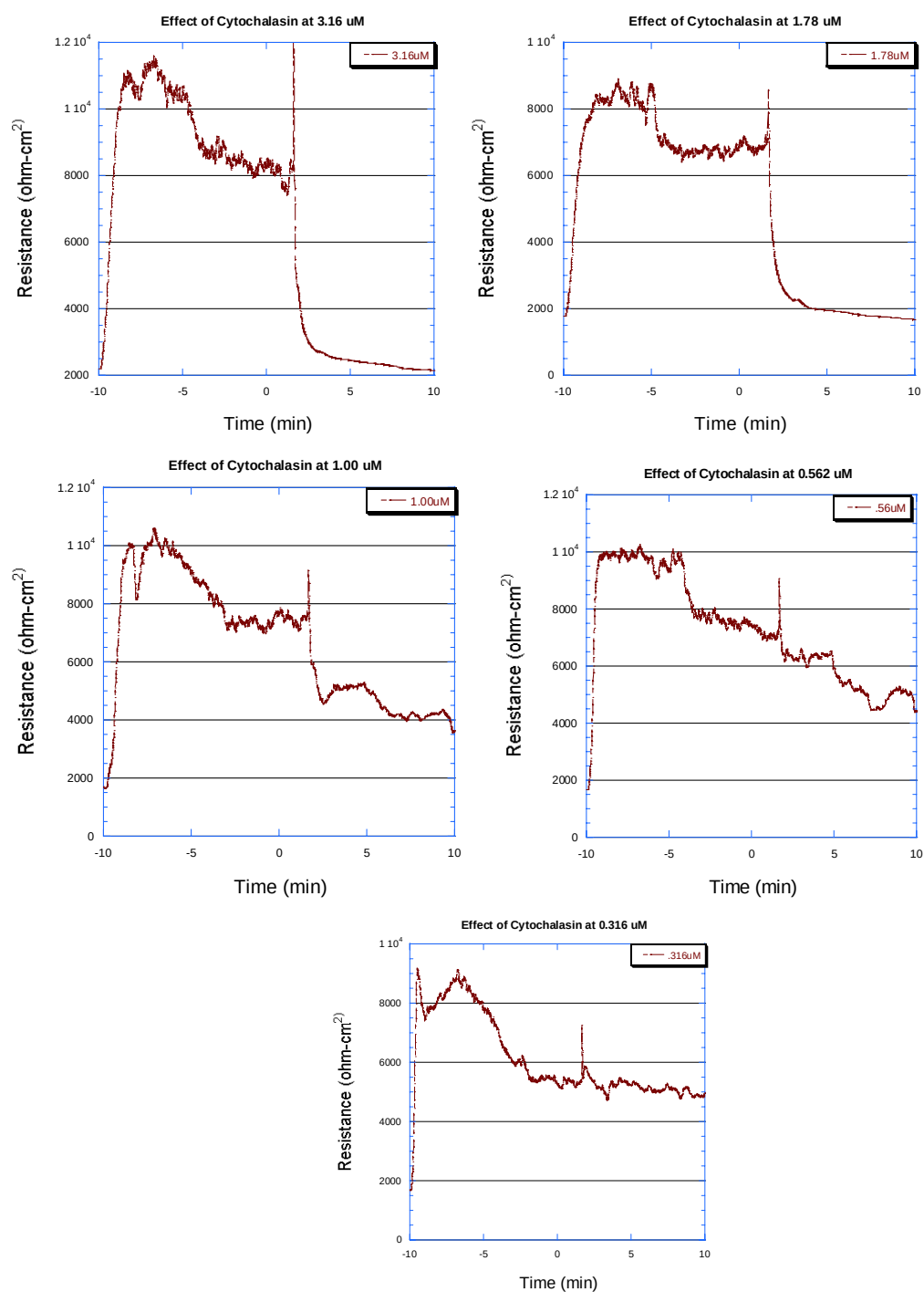


Figure A.1.22: The figure represents the data series obtained at a frequency of 5.62 KHz and treating the cells with concentrations of cytochalasin logarithmically spaced between 3.16 μ M and 0.316 μ M.

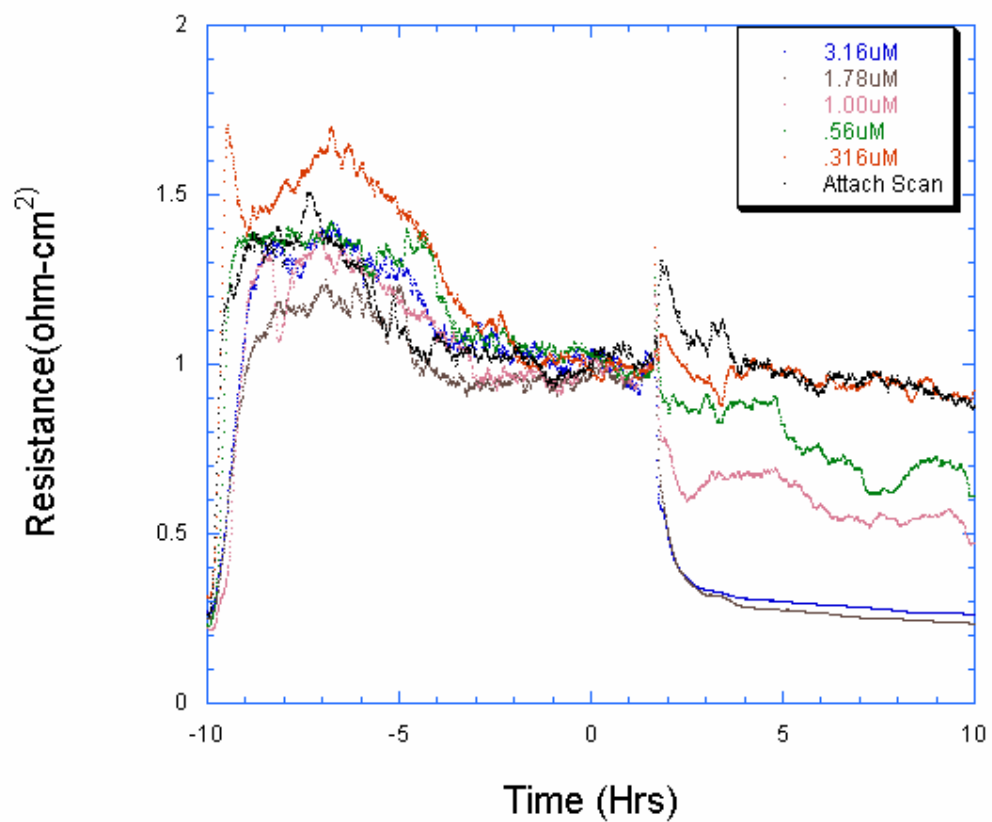


Figure A.1.23: This figure is represented so as to give the user a better understanding of the biosensor in effectively following the intracellular mechanics for different concentrations of Cytochalasin D

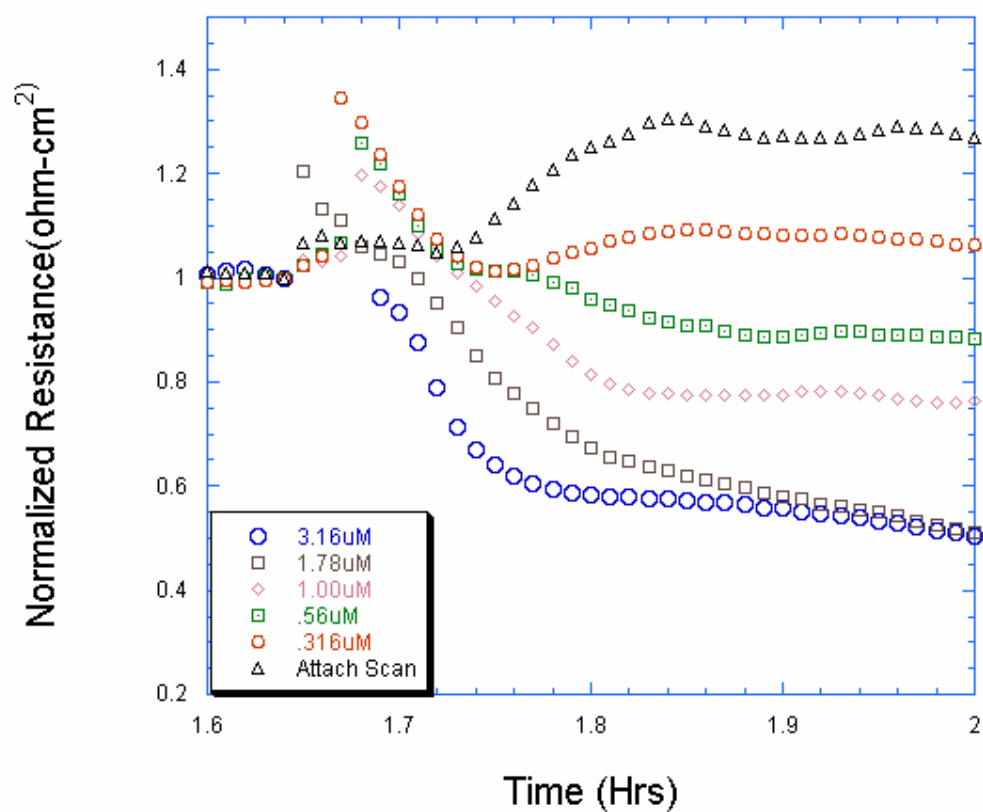


Figure A.1.24: The Normalized data for the actin cytotoxicity assay zoomed to the point just after the addition of the drug to given an idea about the reaction kinetics for the different concentrations

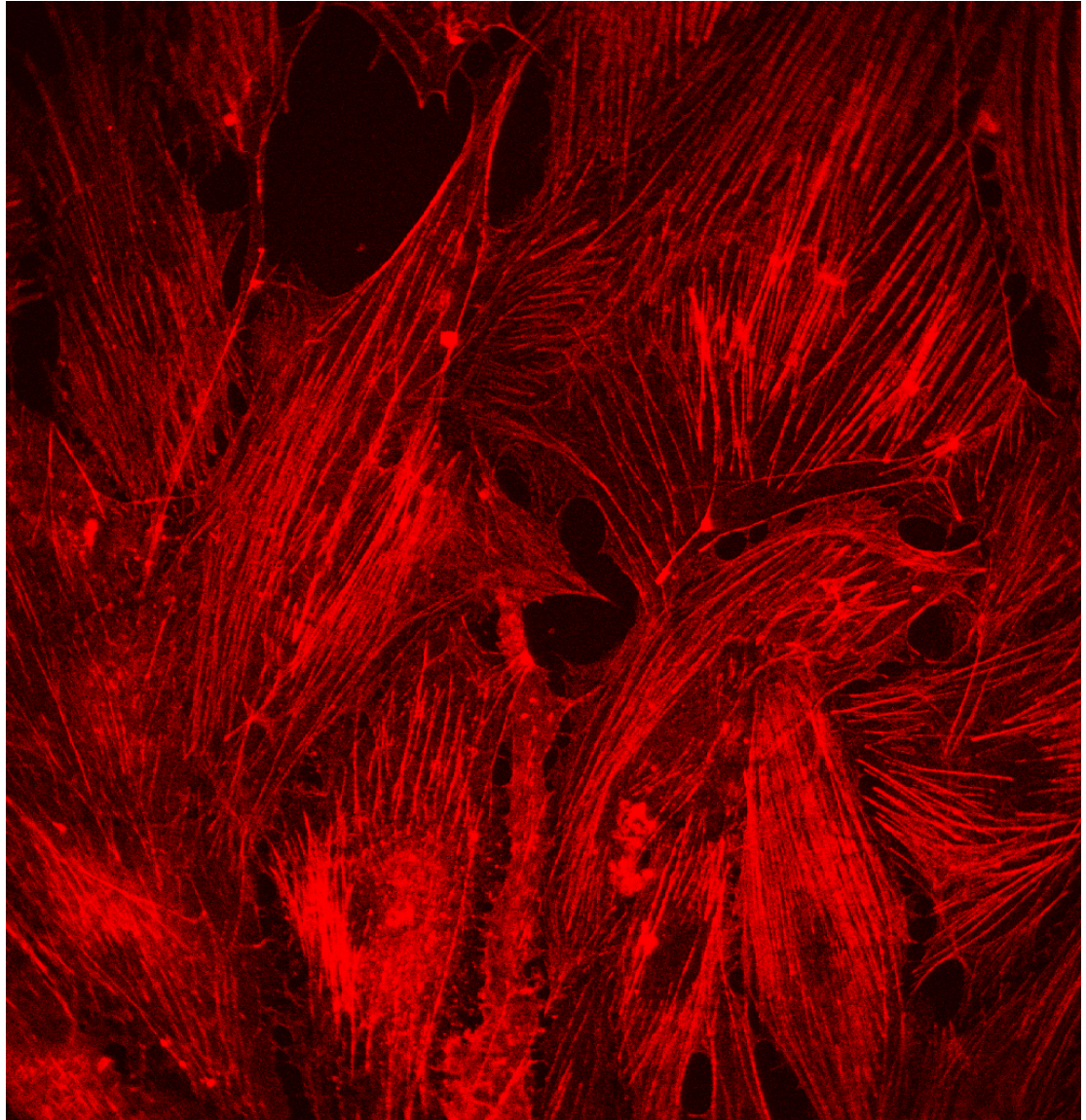
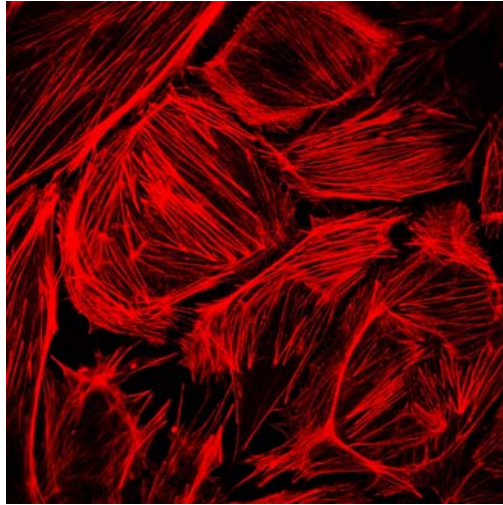
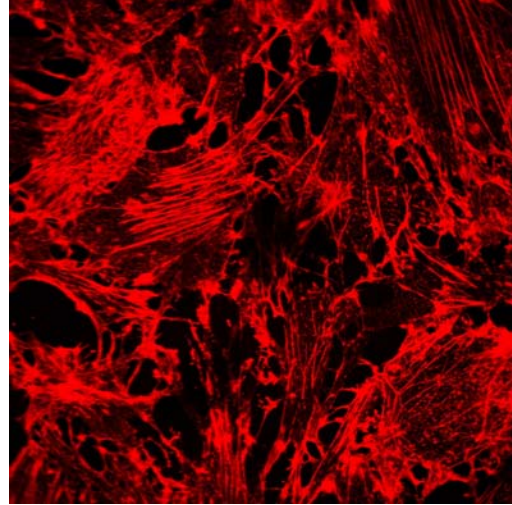


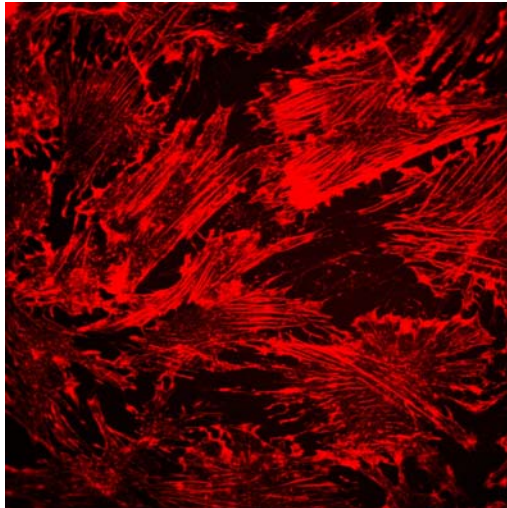
Figure A.1.25: The image is that of an actin stained endothelial cell obtained using the laser confocal microscopy technique. The individual filaments being clearly visible reflects the good staining protocol followed



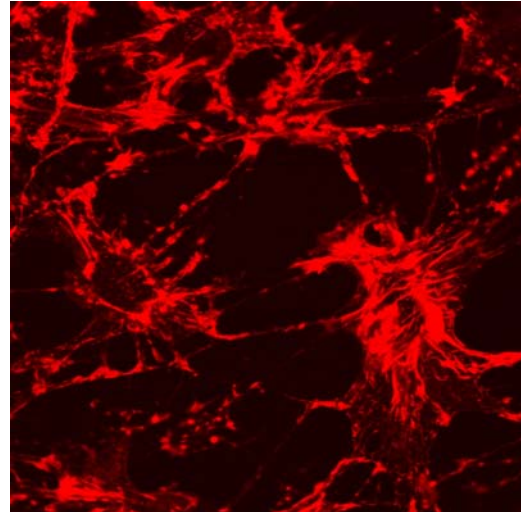
(a) 0.562 μM



(b) 1.00 μM



(c) 1.78 μM



(d) 3.16 μM

Figure A.1.26: A series of confocal images that show the actin disruption for each of the drug concentration used. The filament density can be easily correlated with the drug concentration and this in turn can be correlated with the acquired biosensor data.

The confocal microscopy technique is used here as a means of quantifying the impedance data with respect to the drug concentration

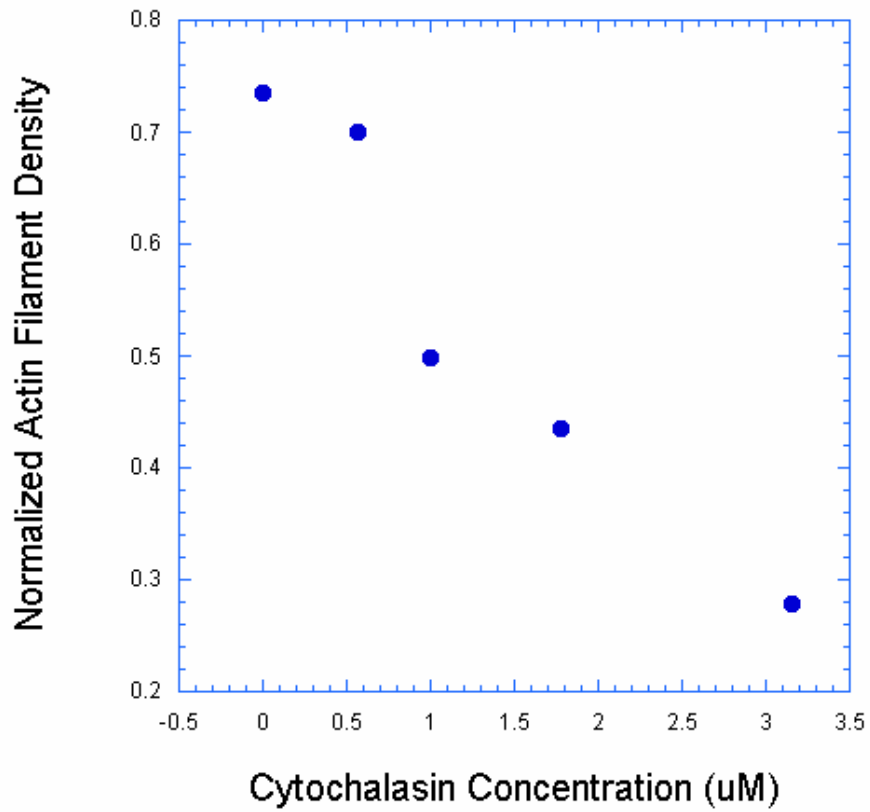


Figure A.1.27: A graph of the actin filament density plotted against the drug concentration. The filament density is obtained by applying standard image processing techniques by programming in Matlab

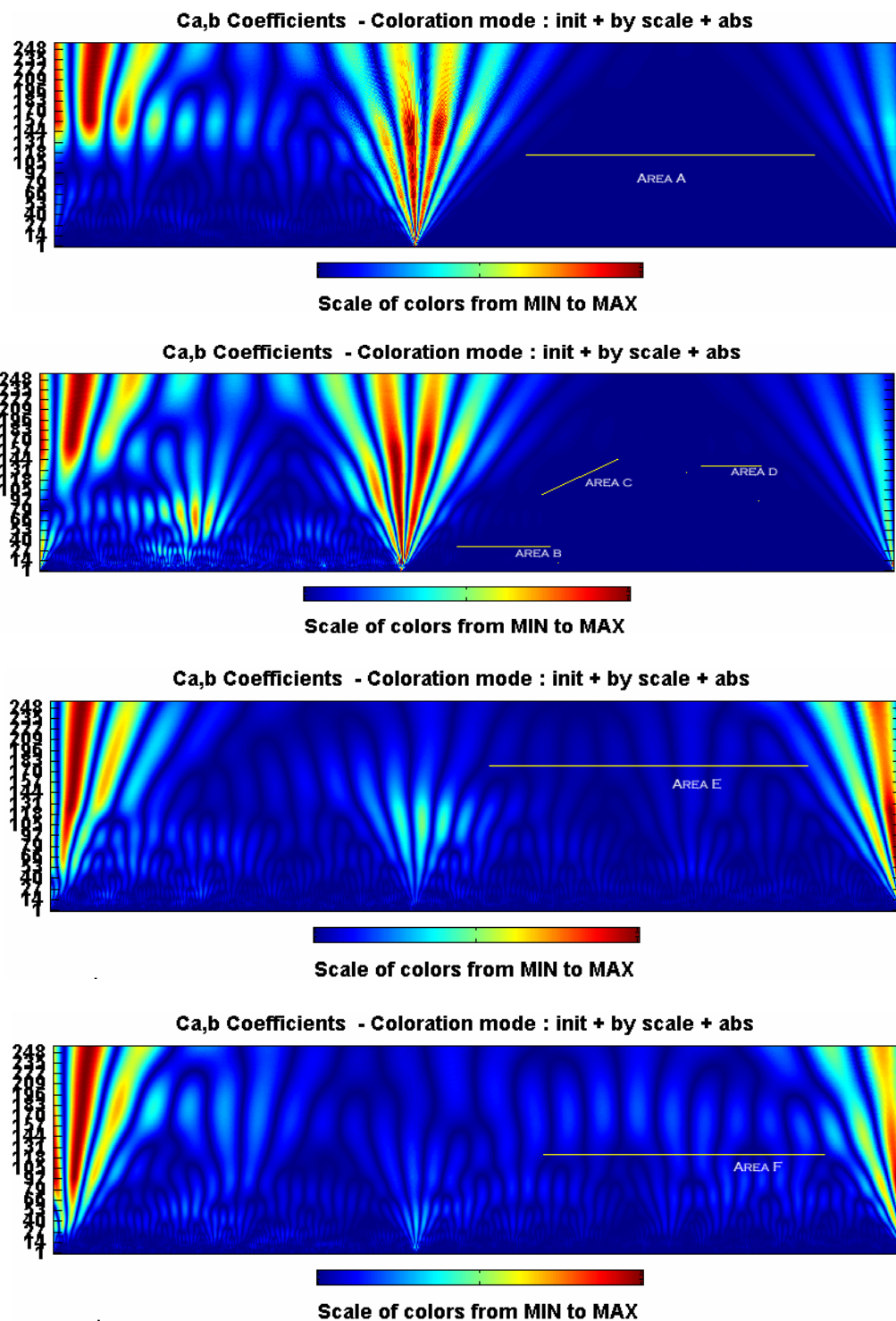
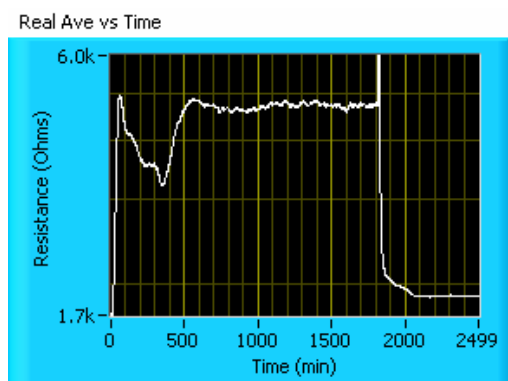
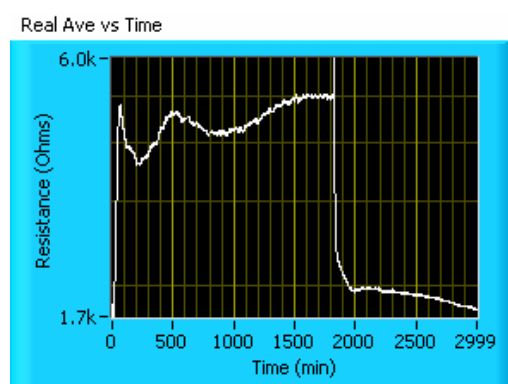


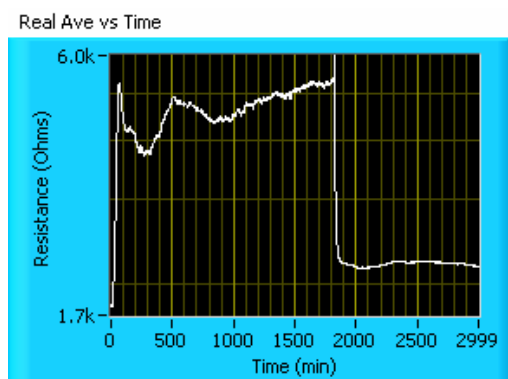
Figure A.1.28 (top to bottom): The wavelet transformed representation of the acquired data for concentrations of 3.16 μM , 1.78 μM , 0.562 μM and the attach scan. The various area markings reveal the cellular response as explained in the discussion.



(a) 100 μM



(b) 56.2 μM



(c) 10 μM

Figure A.1.29: The figure represents the initial time series data obtained for the nocodazole cytotoxicity assay at concentrations of 100 μM , 56.2 μM , and 10 μM . We got a similar cellular response for all concentrations above 10 μM .

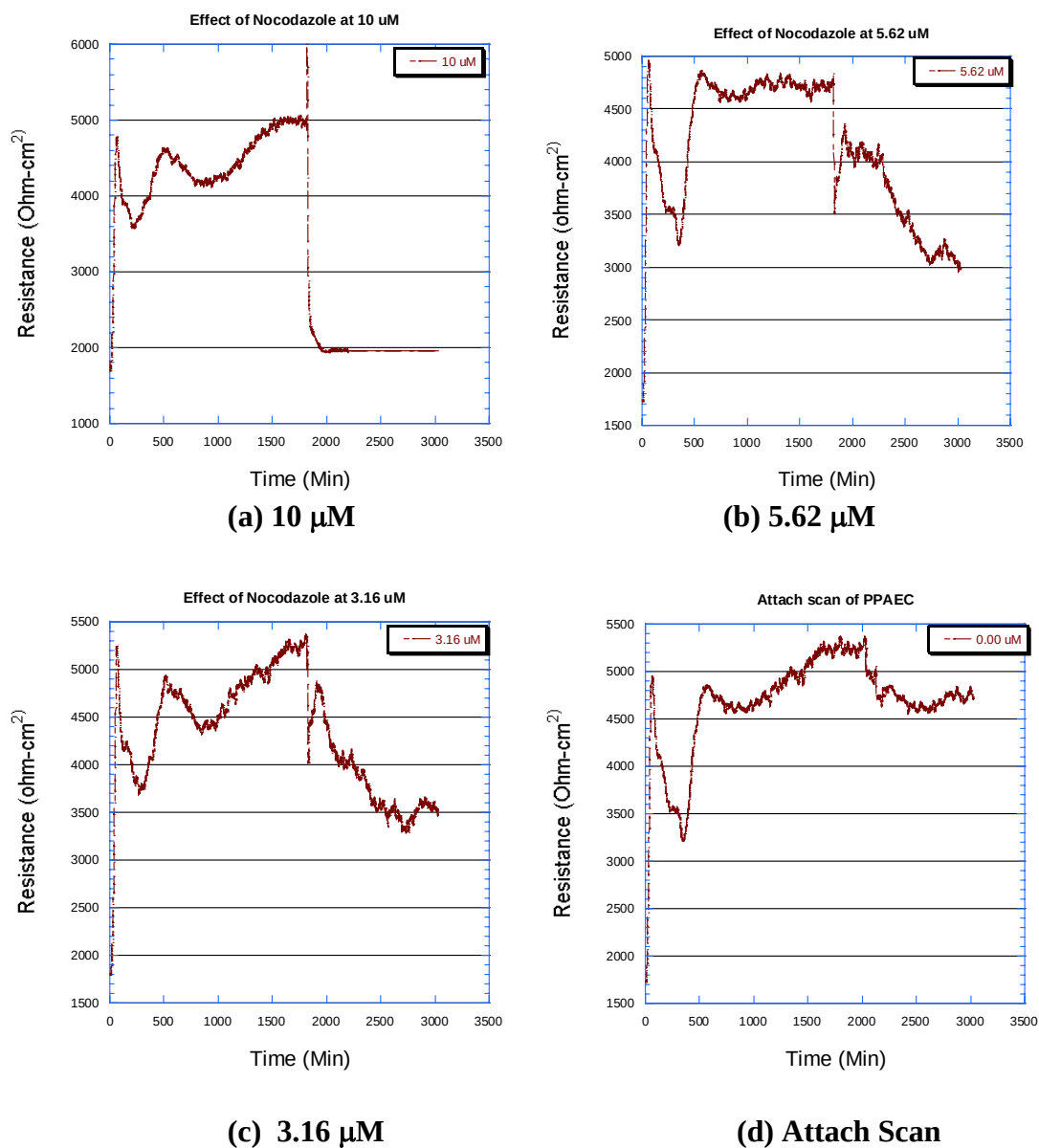


Figure A.1.30: The series of graphs represent the data acquired after treating the cells at modified concentrations ranging between 3.16 μ M-10 μ M

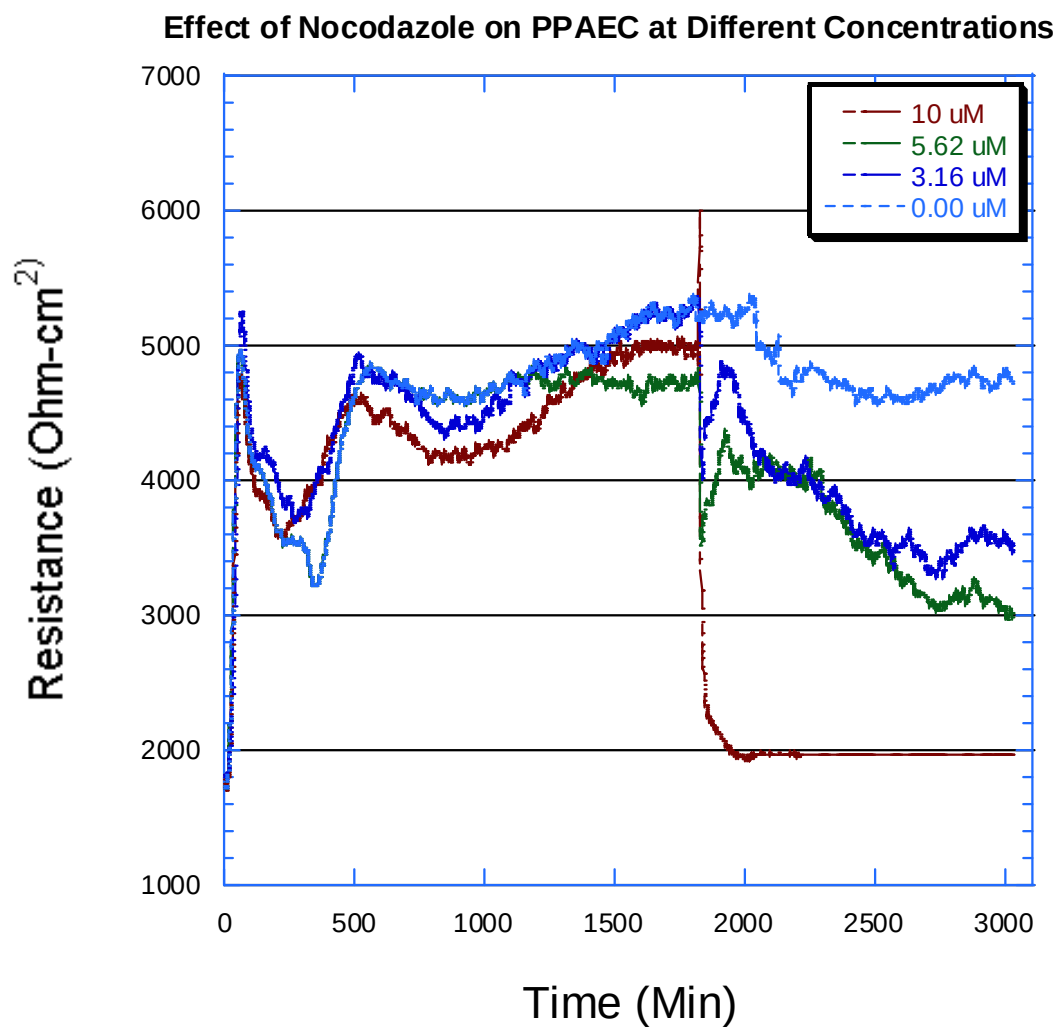


Figure A.1.31: The collective data representing the response of PPAEC to Nocodazole at concentrations mentioned above. This is to give a better understanding of the reaction kinetics at different concentrations of nocodazole

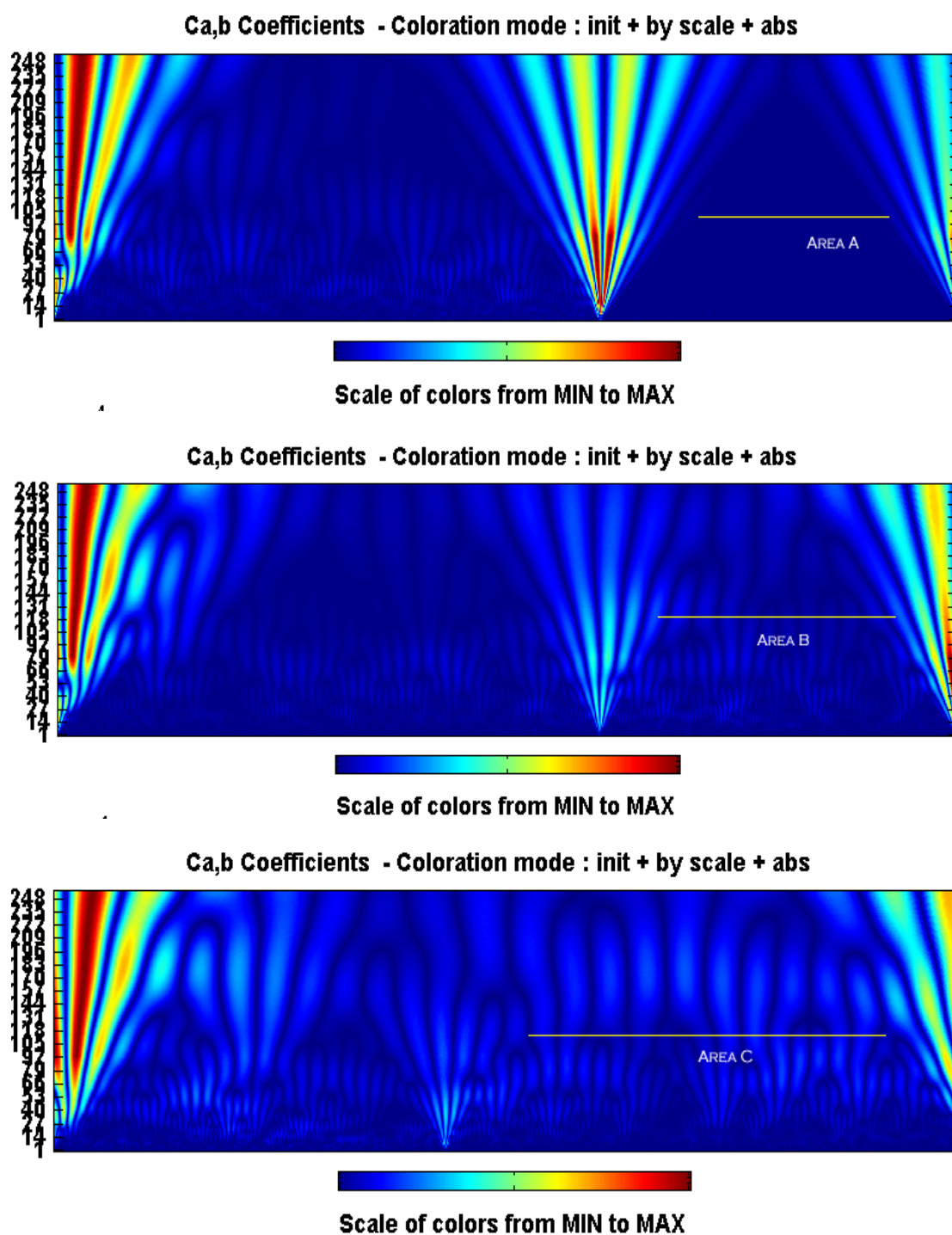


Figure A.1.32: The figures (top to bottom) represent the wavelet transformed data obtained for concentrations of 10 μM , 5.62 μM and the attach scan respectively. The interpretation is similar to that of the wavelet transform for Cytochalasin D data.

Comparison of the effect of Nocodazole and Cytochalasin D at 3.16 μM

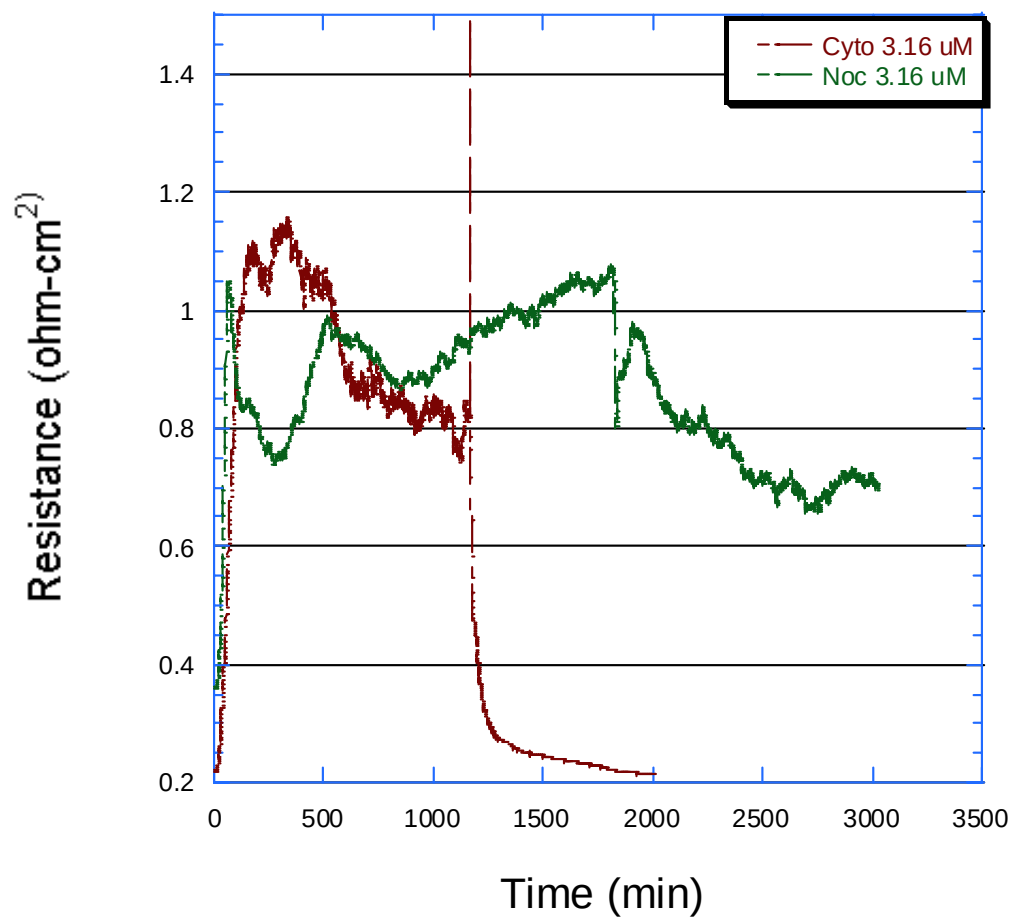


Figure A.1.33: The figure represents the difference in response of the cellular cytoskeleton to different drugs. The varying response detected by the sensor for the same concentration of different drugs is represented here.

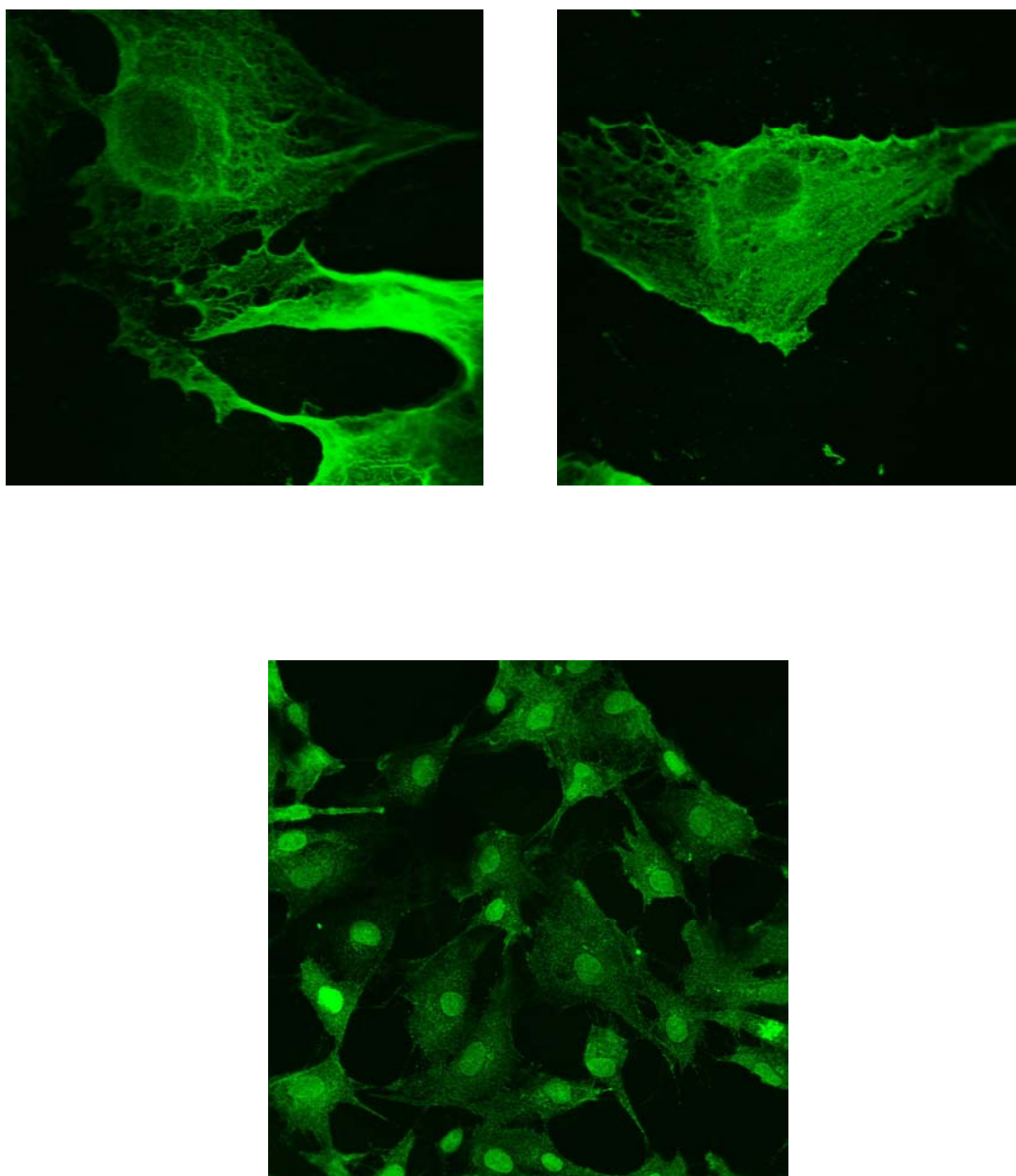


Figure A.1.34: A series of confocal images obtained while attempting to visualize the microtubules. It can be seen that the tubules are not clearly visible as the staining protocol is still under optimization

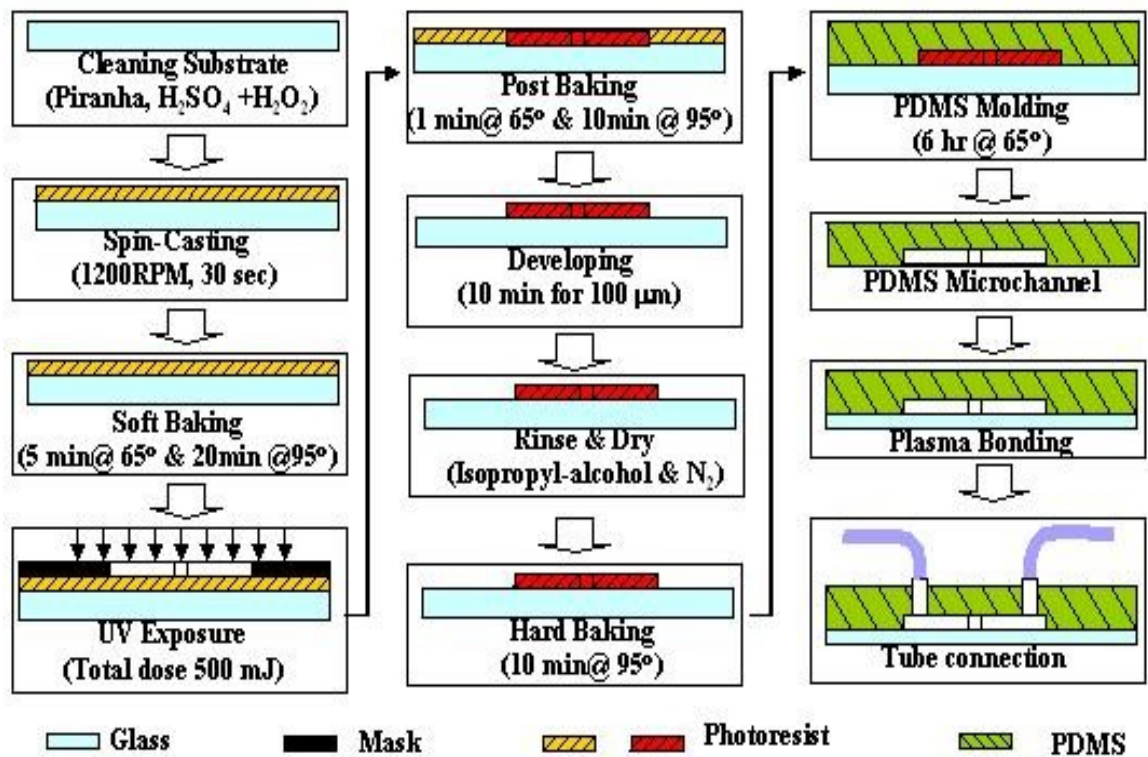
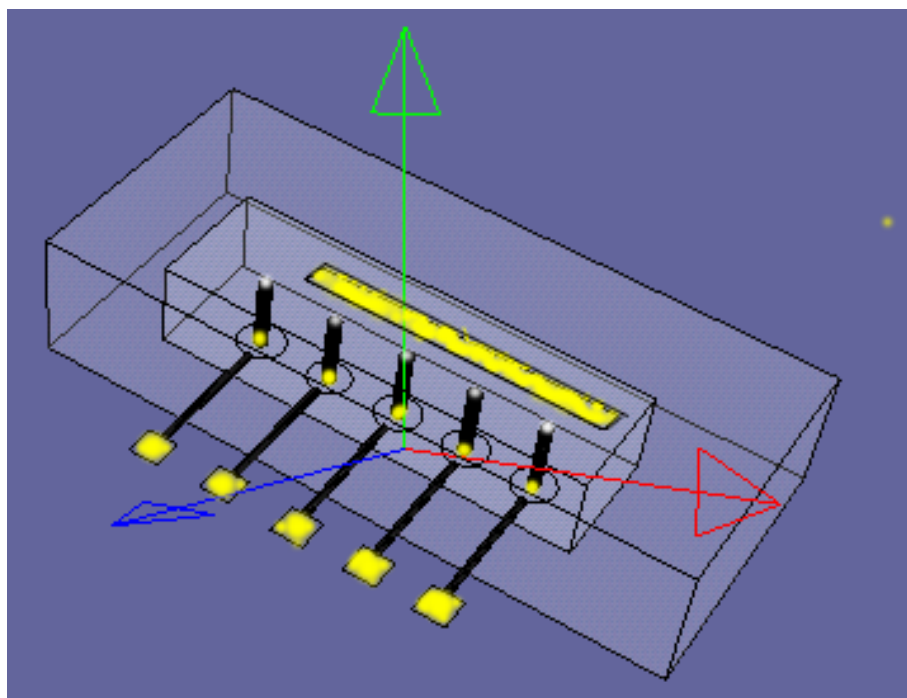


Figure A.1.35: The figure represents the flow process in the design of a microchannel. This is the proposed process for the PDMS based impedance biosensor.

Perspective view:



Side View

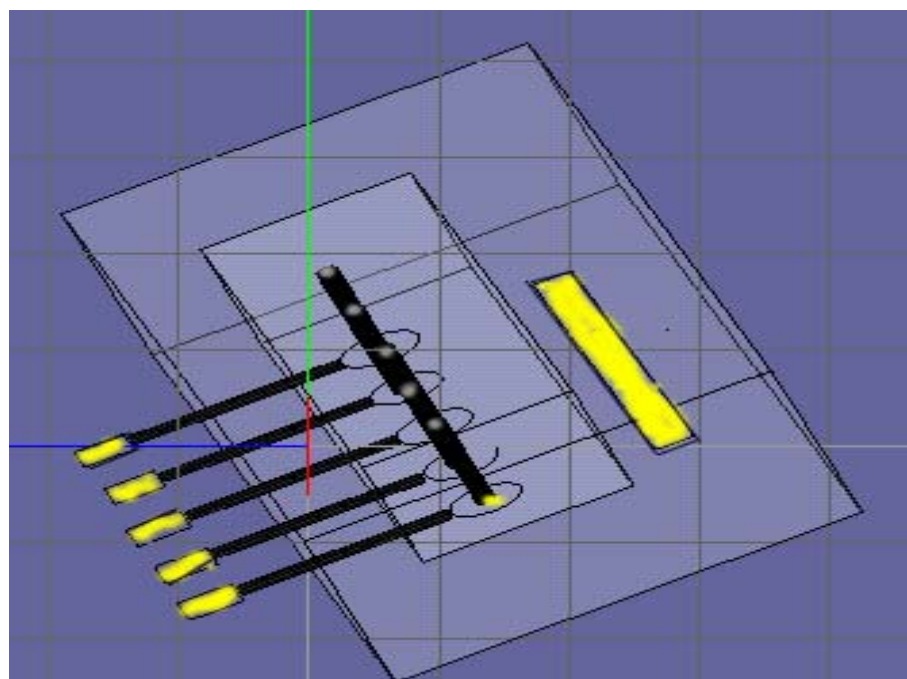


Figure A.1.36: The figure represents the perspective views of the proposed PDMS based cellular impedance biosensor. This arrangement can be used to monitor the cellular activity on artificial surfaces such as hydrogels

Appendix II : Programs and Tables

Matlab and LabVIEW programs

- (i) The Matlab Code used to calculate the cytoskeletal filament density and quantify it as a function of the drug concentration

```
clear all;
close all;
clc;
i1=(imread('actin3.jpg'));% Cell image without any drug
i2=imread('actindrug11.jpg');% image of the cell attacked with
the highest drug concentration(3.16uM)
i3=imread('actindrug21.jpg')% Cell With drug concentration of
1.78uM
i4=imread('actindrug33.jpg')%Cell with drug Concentration of
1.00uM
i5=imread('actindrug41.jpg');% image of the cell attacked with
the least drug concentration(0.562uM)
% the following commands are needed if the images are in RGB
format
j1=rgb2gray(i1);
j2=rgb2gray(i2);
j3=rgb2gray(i3);
j4=rgb2gray(i4);
j5=rgb2gray(i5);
% the following commands are used to generate the filter and
point spread
% function
f=fspecial('motion',10,10);
PSF = fspecial('motion',7,10);
% image is filtered to ge a smoothened version
c1=imcrop(j1);c2=imcrop(j2);c3=imcrop(j3);c4=imcrop(j4);c5=imcrop
(j5);% the image size is cropped so as to demonstrate the
Deblurring Operation
% all the Input cropped images are filtered using the motion
filter.
```

```

f1=imfilter(c1,f);
f2=imfilter(c2,f);
f3=imfilter(c3,f);
f4=imfilter(c4,f);
f5=imfilter(c5,f);
PSF1=ones(size(PSF)); % the Intial PSF guess for the Blind
Deconvolution method
% the following function is used to reduce the ripple effects
occurring
% after image deblurring
% the image is deconvolved to get rid of the blurring effect
it1=edgetaper(f1,PSF);% this function is used to get rid of the
ripple effects
[I1 P1] = deconvblind(it1,PSF1,10);
figure,imshow(f1);title('Original Image Filtered');
figure,imshow(I1);title('Deblurred Image');
it2=edgetaper(f2,PSF);
[I2 P2] = deconvblind(it2,PSF1,10);
figure,imshow(f2);title('Cell Image with Higest Drug
Concentration: 3.16uM');
figure,imshow(I2);title('Deblurred Image ');
it3=edgetaper(f3,PSF);
[I3 P3] = deconvblind(it3,PSF1,10);
figure,imshow(f3);title('Cell Image with Drug Concentration:
1.78uM');
figure,imshow(I3);title('Deblurred Image ');
it4=edgetaper(f4,PSF);
[I4 P4] = deconvblind(it4,PSF1,10);
figure,imshow(f4);title('Cell Image with Drug Concentration:
1.00uM');
figure,imshow(I4);title('Deblurred Image ');
it5=edgetaper(f5,PSF);
[I5 P3] = deconvblind(it5,PSF1,10);

```

```

figure,imshow(f5);title('Cell Image with Drug Concentration:
0.562uM');
figure,imshow(I5);title('Deblurred Image ');

% HISTEQ is performed depending upon the image contrast
h1=histeq(I1);
h2=histeq(I5);
% the other images are good enough that the histogram
Equalization is not
% necessary
% the threshold is found out by trial and error and an optimum
value of
% 0.3 is found and applied to all the images
bw1=im2bw(h1,.3);
bw2=im2bw(I2,.3);
bw3=im2bw(I3,.3);
bw4=im2bw(I4,.3);
bw5=im2bw(h2,.3);
% the BWLABEL function is used to label the objects in the
image.
% the importance of this function is that it also separates the
background
% from the objects
l1=bwlabel(bw1,8);
l2=bwlabel(bw2,8);
l3=bwlabel(bw3,8);
l4=bwlabel(bw4,8);
l5=bwlabel(bw5,8);
% the region props command is used to find out the area of the
objects in
% the image. here it gives the area covered by the cells for
each and
% every drug concentration

```

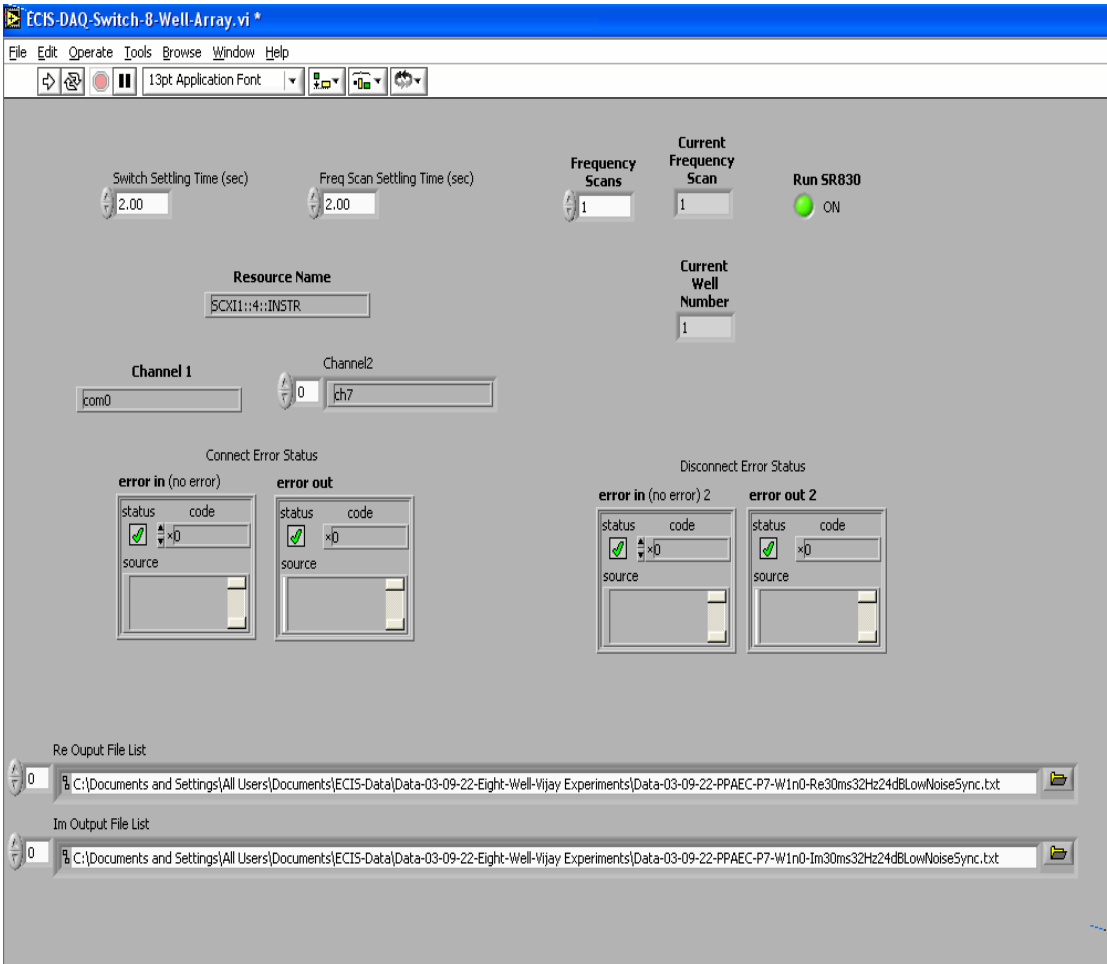
```

reg1=regionprops(l1,'area');
reg2=regionprops(l2,'area');
reg3=regionprops(l3,'area');
reg4=regionprops(l4,'area');
reg5=regionprops(l5,'area');
% The total sum of the areas covered by the cells for each case
is calculated
t1=sum([reg1.Area]);
t2=sum([reg2.Area]);
t3=sum([reg3.Area]);
t4=sum([reg4.Area]);
t5=sum([reg5.Area]);
% the following command is used to calculate the total image
area which
% includes both the cells and the background
imt1=regionprops(h1,'area');
imt2=regionprops(I2,'area');
imt3=regionprops(I3,'area');
imt4=regionprops(I4,'area');
imt4=regionprops(h2,'area');
ima1=sum([imt1.Area]);
ima2=sum([imt2.Area]);
ima3=sum([imt3.Area]);
ima4=sum([imt4.Area]);
ima5=sum([imt5.Area]);
% now the ratio of the area covered by the cells to the total
image area
% is calculated and this varies as a function of the druh
concentration.
% for the highest drug concentration, this ratio is the least
and
% increases as the drug concentration decreases.
r1=t1/ima1;

```

```
r2=t2/ima2;  
r3=t3/ima3;  
r4=t4/ima4;  
r5=t5/ima5;
```

(ii) A screen shot of the Virtual Instrumentation program used to control the biosensor instrumentation and to acquire data



Tables

- (i) Cytoskeleton Filament density obtained through image processing techniques in Matlab and the corresponding concentrations

Drug Concentration	Area covered by cellular filaments	Total Image Area	Cell Area Ratio
3.16 uM	288674	1048576	0.2753
1.78 uM	445667	1048576	0.4250
1.00 uM	494440	1048576	0.48
0.562 uM	744788	1048576	0.7206
000 uM	765670*	1048576	0.7310

(ii) Spin rate of the Spin coder and the corresponding thickness of the photo-resist that would be obtained

<u>Product</u>	<u>Thickness(μm)</u>	<u>Spin Speed(RPM)</u>
<u>SU-8 2035</u>	<u>35</u>	<u>3000</u>
	<u>55</u>	<u>2000</u>
	<u>110</u>	<u>1000</u>
<u>SU-8 2050</u>	<u>50</u>	<u>3000</u>
	<u>75</u>	<u>2000</u>
	<u>165</u>	<u>1000</u>
<u>SU-8 2075</u>	<u>75</u>	<u>3000</u>
	<u>110</u>	<u>2000</u>
	<u>225</u>	<u>1000</u>
<u>SU-8 2100</u>	<u>100</u>	<u>3000</u>
	<u>140</u>	<u>2000</u>
	<u>260</u>	<u>1000</u>

Vita

Vijay Nandakumar was born July the 30th , 1982 in Tanjore, India. He graduated from the University of Madras with a Bachelor of Engineering in Electrical Engineering. He came to the University of Tennessee to pursue a M.S. in Engineering Science with a Biomedical emphasis. Vijay is currently looking for a position in the Bio-Engineering industry.