



Novel Technologies for Cell Culture and Tissue Engineering

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Submitted to the University of Oxford for the degree of
Doctor of Philosophy

Institute of Biomedical Engineering
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July 2017

Abstract

Cell culture has been a fundamental tool for the study of cell biology, tissue engineering, stem cell technology and biotechnology in general. It becomes more and more important to have a well-defined physiochemical microenvironment during cell culture. Conventional cell cultures employ expensive, manually controlled incubation equipment, making it difficult to maximize a cultures yield. Furthermore, previous studies use qualitative methods to assess cell culture proliferation that are inherently inaccurate and labour intensive, thereby increasing the cost of production. In addition, three dimensional cell culture, in scaffold, has been shown to provide more physiological relevant information as it mimic more accurate conditions that are similar to the physiological conditions of the human body compared with two dimension, which has special interest to regenerative medicine.

Therefore, a portable and automated total-analysis-system (μ TAS) was proposed with microenvironment control and quantitative analysis techniques to monitor cell proliferation and metabolic activity. The automated portable heating system was validated to be capable to maintain a stable physiochemical microenvironment, with little margin of error, for cellular substrate outside of conventional incubation. A standalone platform system was designed and fabricated with accurate temperature control by employing an optically transparent ITO-film with a large heating area. The transparency of the film is critical for continuous in-situ microscopic observation over long-term cell culture process. Previous studies have attempted to use ITO-film as a heating element, but were unable to distribute the heat evenly onto the microbioreactor platform. This nagging problem in the literature was improved through a novel film design. As a result, the ITO-film based heating system was evaluated and constructed successfully to serve as a heating element for long-term static cell culture with facilitated proliferation rate in gas-permeable PDMS microbioreactor outside of conventional incubation.

In addition to maintaining a stable microenvironment, a non-invasive in-situ technology for monitoring cell viability and proliferation rate was constructed and developed based on bioimpedance spectroscopy (BIS). It was primarily focused on making decisions for structure and specification of proposed system-on a chip BIS measurement. The miniaturization of BIS system on microbioreactor platform was achieved by utilizing and integrating switching matrix array, impedance analyzer chip with reliable analogue-front-end circuitry. The realized system was verified with the DLD-1 cells and its monitored data were validated with conventional bioassays.

Three dimensional cell cultures with scaffold is a key to the success of tissue engineering. Engineered cornea collagen scaffold may be feasible using re-seeding proper human cells onto a decellularized corneal scaffold. The quality of the scaffold and the interaction of the cells are critical to the key function (i.e transparency, haze and total transmittance) of final products. An integrated corneal collagen scaffold quality assessment system, via optical property inspection unit, was innovatively designed and realized with non-invasive and non-destructive characteristics. The H1299 cells were seeded onto inspected corneal scaffold and BIS system, which were realized in the previous chapter, were used to validate its applicability for 3D cell culture. The cell adhesion as an outcome at different scaffolds with different optical properties has revealed the importance of the microstructure of scaffold on the cell functions.

The results showed the developed technologies can be used for the quality control of corneal scaffold and the fabricated μ TAS not only enabled environmental control but, with BIS-based in-situ assay, it also facilitate the function (i.e adhesion) and viability monitoring with quantitative and qualitative analysis in 3D cell culture. Additionally, by considering its low decontamination and cost-effective nature with compatibility for high-throughput screening applications, the fabricated and integrated systems has significant applications in tissue engineering.

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Symbols

A	area	cm^2
a_i	activity of species i	mol cm^{-3} or mol dm^{-3} (M)
C_{DL}	double layer capacitance	$\mu\text{F cm}^{-2}$
C_m	membrane capacitance	$\mu\text{F cm}^{-2}$
D_i	diffusion coefficient of bulk species i	$\text{cm}^2 \text{s}^{-1}$
E	electrode potential	V
ΔE	difference in sequential formal potentials	V
f_c	characteristic frequency	Hz
I	ionic strength	mol cm^{-3}
I_p	peak current	A
I_{ss}	steady-state current	A
I_o	Output current	A
j_o	flux	$\text{mol s}^{-1} \text{cm}^{-2}$
q or Q	charge passed	C
R_e	resistance of extracellular	Ω
R_i	resistance of intracellular	Ω
R_m	resistance of membrane	Ω
t	time	s
T	temperature	K
V_f	volume flow rate	$\text{cm}^3 \text{s}^{-1}$
v_o	solution velocity through the centre of electrode	cm s^{-1}
v_x	solution axial velocity	cm s^{-1}
x_c	reactance	Ω
σ_r	relative Conductivity of species	μSCm^{-1}
ε_r	relative permittivity of species i	F/m
ε_0	vacuum permittivity of species i	F/m
ρ_r	resistivity of species	dimensionless
α, β	Relaxation Dispersion Range	dimensionless
$V_{measure}$	measured bioimpedance	Ω
ω	angular frequency	Hz
μ	micro-	
τ	dimensionless time parameter	
Σ	sum of	

Abbreviations

BIS	B io I mpedance S pectroscopy
C2VC	V oltage t o C urrent C onverter
CCD	C harge C oupled D evice
CLT	C entral L imit T heorem
CMRR	C ommon M ode R ejection R atio
CV	C yclic V oltametry
DMEM	D ulbecco's M odified E agle's M edium
ECF	E xtracellular F luid
ECIS	E lectric C ell- S ubstrate I mpedance S ensing
ECM	E xtra C ellular M atrix
EDTA	E thylene- d iaminete- t raacetic A cid
EIT	E lectric I mpedance T omography
ETHD-1	E thidium H omodimer-1
FBS	F etal B ovine S erum
GUI	G raphical U ser I nterface
I²C	I nter- I ntegrated C ircuit
ICM	I ntra C ellular F luid
IEC	I nternational E lectrotechnical C ommission
IR	I nfra R ed
ISO	I nternational for O rganization S tandardization
ITO	I ndium T in O xide
MPM	M ulti P hoton M icroscope
PBS	P hosphate B uffered S aline
PCB	P rinted C ircuit B oard
PDMS	P oly- d i- m ethyl- s iloxane
PID	P roportional I ntegral D erivative
SF-BIA	S ingle F requency B ioimpedance A nalysis
SHG	S econd H armonic G eneration
SNR	S ignal-to N oise R atio
SSIM	S tructural S imilarity I ndex M ethod
μTAS	M icro T otal A nalysis S ystem
UV	U ltra V iolet
V2CC	V oltage t o C urrent C onverter

Acknowledgements

Firstly, I would like to take this opportunity to express my sincere thanks to my supervisor, Professor Zhanfeng Cui (Fellow of the Royal Academy of Engineering), for accepting me into the Tissue Engineering and Bioprocessing Group at Oxford. Without his patient guidance, timely support and great kindness, I would not have made it this far. I would also like to thank Dr Julian George, Dr Shaoyang Yeh and Professor Cathy Ye who have introduced me to cell culture techniques in the field of biology. Their expertise in this field has spurred me on in my research.

This thesis is the result of collaborations between a few Universities and Institutions. The thermal distribution on ITO-film and microbioreactors were analysed via Infrared Thermography at Hamlyn Biomedical Centre at Imperial College. The stained cells expressed in mCherry fluorescent protein were provided by Dr Julian George with David Nagel from Aston University. Furthermore, the genetically modified cancer cell H1299 with removal of RASSF1A gene expression was confirmed and provided by Dr Xiao Wan and her colleague in CRUK/MRC Oxford Institute of Radiation Oncology Department. The Cyclic Voltammetry analysis was carried out in the Practice and Theoretical Chemistry Laboratory Oxford University. Moreover, part of the prototype investigation and integration of proposed non-invasive and non-destructive Collagen Corneal Scaffold Inspection system took place in Basingstoke with close collaboration from LG Motion Ltd and Scorpion Vision Ltd.

I would like to say how honoured, grateful and lucky I am to have such thoughtful parents offering me patience and kindness, invaluable advices and devotions. Moreover my wife, Dr Qian Li, has always been there for me with gentle support and encouragement. whenever I needed it. Our shared joy and happiness has inevitably enriched research life at Oxford.

All work and no play makes Jack a dull boy, not everything in life is work. I would like to specially thank my close friends who have made my time at Oxford so memorable. The naughty Dr Atsushi Takagi, always being energetic and creative; the passive and expressive Dr Wassif Shabbir; Mr Joshua Parker surprisingly matured in those years, particularly after joining the Department of Energy in Central Government. All of them have corrected my English a great deal over the past years. Additionally, Mr Neil Murray undoubtedly increased his enthusiasm for Guinness in the isolated village, of Gaulby. In the affluent student life at Oxford, a team was formed with Dr Xuan Zhang and Dr Danni Zhu through intensive society events;. Lastly, the warm hospitality with authentic food prepared by Mr Xiaoyu Chen and Ms Juan Zhang in Reading cured my homesickness.

There is an old expression in Chinese that one should always think of the source when drinking still water. Hence, I would like to express my sincerest thanks to my old mentors: Dr Nir Grossman, joint Fellow at MIT and Harvard Medical School, and Professor Christofer Toumazou (FREng, FRS, FMedSci) from Imperial, who initially introduced me to the Bioimpedance Spectroscopy field a few years ago. Moreover, Professor Zihe

Rao, Fellow of the Chinese Academy of Science, Professor Yu Wei, Fellow of the Chinese Academy of Engineering, and Professor Sir Keith ONion (FRS) have kindly provided me with opportunities that have broadened my vision whilst showing determinations and perseverances in their own research, truly inspiring me.

To make a D.Phil, in addition to formally meet the requirements includes brought novelty, patent realization and thesis submission, the process of itself should also being an exploration and investigation journey for core values. The deepest gratitude with greatest appreciation therefore goes to my bosom friends including the only Chinese director of History at Harvard-Yenching Institute, Professor Weiming Tu, Professor Kang Li and Professor Bo Liu, who were both Professors of Philosophy and Sociology in China.

It was Professor Liu who explained to me, just before I officially join Oxford, that Higher Education, to a certain extend, has evolved from a tradition of aristocratic cultivation to teaching skills in secular society. As a result, the barriers between subjects and fields had growing larger and larger which not only separated the past history and experiences of undergoing life, but also isolated the world and society we share. As the education being converted to a subject teaching and technical training oriented event, when faces life it can only make people to despise the higher education itself and, as a result, the life and higher education became so fragmented without sufficient interconnections.

I was lucky enough to meet Professor Kang Li, a senior academic visiting scholar from Peking University, who introduced the German Philosopher Max Webers theory to me during my D.Phil studies at Oxford. He made me believe the judgement of actions (i.e to pursue a D.Phil) should not only depend on its success value (Erfolgswert) but more importantly should be consistent with its convictional value (Gesinnungswert). In other words, the process of pursuing the D.Phil should not only be focused on solving the practical issues based on scientific-driven engineering-realized procedures. It may not even be necessary to be obliged to resolve the fundamental theoretical propositions, but more critically, to perceive the intentions of individual life course, ultimate concern and hence the internal association between academic tradition and current events.

This methodology was inspirationally (for me) hinted at by Professor Weiming Tu, Fellow of the American Academy of Arts and Sciences: the only way to discover the convictional value of a D.Phil in Oxford is to actively and consciously learn through the implementations of a D.Phil since hearing without seeing; though learned, is false; seeing without knowing; though memorized, is mistaken; knowing without practicing, though profound, is confusing. With helps from the above mentioned family members, friends and mentors, the D.Phil at Oxford has indeed shaped the way I experience the everyday world and accurately reflects the meaning of the word Theory in Greek: a gaze, a way of seeing.

Chapter 1

Introduction

1.1 Scope of the Thesis

Regenerative medicine usually covers tissue engineering and stem cell therapy. Living and functional cells are the core of the therapeutic approach. Cell culture is then a critical step in the development of the new therapeutic and also in general cell biology research and general biotechnology.

Over the past decade there have been enormous advances in the field of tissue engineering and is becoming one of the most important areas of regenerative medical science for the foreseeable future. Tissue engineering can be defined as an interdisciplinary field that utilizes the principles of emerging engineering and biotechnology to develop functional tissues in a laboratory setting using cells [Divr 2011] where the related subject is illustrated in Figure 1.1. It collectively includes cellular and tissue biology, engineering realization, materials, biochemical and physiochemical factors inspection and, potentially, tissue implantations. This combination is expected to improve and perhaps replace biological functions [Atala 2004].

Despite the fact that definition for tissue engineering cover a large range of fields and applications, the laboratory process is closely tied with cell/tissue culture. More importantly, culturing tissues/cells in micro-scale devices provides a more holistic model

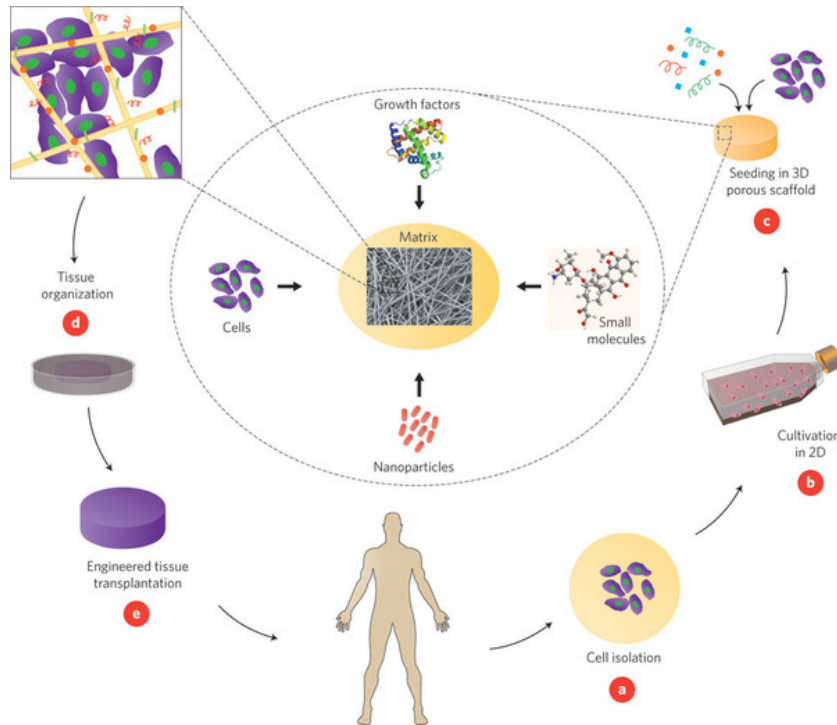


FIGURE 1.1: An Illustration of Tissue Engineering [Dvir 2011]

for identifying cellular response to stimuli and the capacity to reproduce the cellular microenvironment [Barrilleaux 2006], unlike in a macro-scale bioreactor system. In theory, an effective in-vitro cell culturing microbioreactor platform should reliably mimic the in-vivo micro environment of the cell. The identification of cellular behaviour in bioreactor for each type of cell during the proliferation process not only plays an important role in the process of repairing different types of human tissue, but also facilitates the discovery of new drugs and gene function research. Major improvements in the treatment of many problems such as cancer, Parkinsons disease and spinal injuries can be attributed study to relevant types of cells in tissue culture [Naderi 2011].

On the other hand, the stem cells are known to have potency [Jaenisch 2008; Reya 2001] to renew themselves and differentiate into other types of cells [Keenan 2012]. These traits give stem cells an important role in many areas including the process of repairing different types of human tissue, the development of new drugs and gene function research [Barrilleaux 2006]. Though stem cells technology and therapy exploits the unique advantage, the developments of technologies are also largely dependent on cell culture in

micro-scales [Flaim 2008; Moeller 2008].

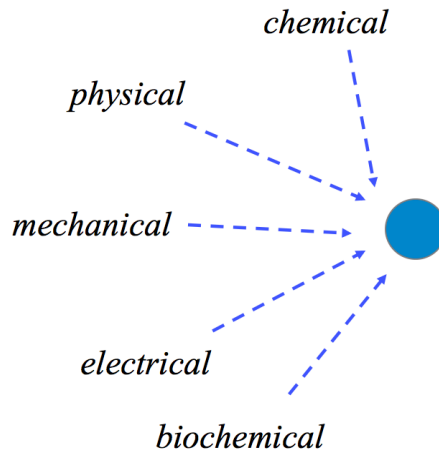


FIGURE 1.2: Illustration of potential effects from micro-environment that may impact on cell culture

Thus, the cell culture in bioreactor has always been an essential laboratory technique for many applications, such as investigation of physiology and pathology of cellular substrate [Wu et al. 2007; Schultz and Lucas 2006; Xu et al. 2006], toxins [Cui et al. 2007] and growth factors of specific cellular substrates. To mimic the microenvironment, the identification and understanding of the cellular microenvironmental impact (also known as the cell niche) is needed. Past research indicates that the success of cell culture is hugely dependent on the physical and chemical environment [Hardman 2006], nutrients and metabolites as well as growth factors and many quantitative controls [El-Ali 2006]. The combination of the complex set of physical, chemical and biological conditions that surround the biological cell is known as the in-vivo cellular microenvironment (illustrated in Figure 1.2) Even a small change in temperature, pH or humidity could have an impact on a cells survival, growth and metabolic state [Burdick 2009; Walker 2004].

For the most types of cell, the physiological temperature of the microenvironment was reportedly needs to be maintained between 35 °C and 38 °C. Nevertheless, some cell

types are more sensitive to changes in temperature than others. Under in-vivo circumstances, the temperature is maintained via the thermal release from collective cellular metabolism, and homoeostasis of the human core temperature is tightly regulated at which all the functions of the human cells can operate with optimal efficiency, and its offset by systemic processes that include shivering and perspiration [Diaz and Becker 2010; Imrie 1990]. If the core temperature rises above 40 °C for an extended period, many of the metabolic and enzymatic processes within these cells become disrupted, and cellular function will be lost. Whilst small period of exposure is offset by the release of a family of heat shock proteins (HSP) that act to stabilise the folding or other key cellular proteins, however, this protection is rather limited [Schlesinger 1990; Ritossa 1962]. Conversely, cell usually accompanies low metabolic energy at temperatures below 35 °C where the enzymatic processes are also slowed and degraded. No effective immune response can be mounted under low core temperature circumstance and human cells become vulnerable to viruses and chronic infections which is considered as a sign of degeneration and gradual cellular death [Liu 1972].

Furthermore, recent literature suggested that the cellular temperature sensing-receptor has great sensitivity and a small variation of micro-environment temperature not only changes the rate and nature of chemical reactions, but also it may alter the configuration of atoms that make up nucleic acids, proteins, lipids and other biomolecules, significantly affecting their activity. Some sensors such as ribonucleic acid (RNA) sensing-receptor are optimized to rapidly and specifically affect a defined process such as altered translation rates of relevant mRNA upon a change in temperature. Conversely, thermosensors such as bacterial cold sensor and transient receptor potential (TRP) channels alter cellular physiology more globally via alteration of membrane fluidity and excitability, respectively. [Sengupta and Garrity 2013] While the molecular mechanisms by which nucleic acid sensors and some bacterial proteins sense temperature are studied extensively, the

sensitivity and mechanisms of other thermosensors remains unclear and will be an important avenue for future investigation. However, the discussion of diversity and sensitivity for biological thermosensors are beyond the scope of this thesis and, in order to maintain a homeostasis microenvironment for temperature sensing receptors, the fluctuation in culture chamber were designated to be strictly controlled within $\pm 0.3^{\circ}\text{C}$.

Other than temperature, physiological processes are also pH sensitive and number of studies had been conducted and shown the dramatic effect on the cellular metabolism and function with different levels of ionic concentration of medium surrounding each cell. Many salts such as Magnesium and Calcium play a fundamental role in both cell attachment and cell behaviour, and changes in the concentration of salts rapidly modifies the processes occurring on the cell surface and inside the cell via ion channels in the cellular membranes [Wu et al. 2007]. The pH value is directly connected to the potential across ion channel which known as membrane potential [Charlwood 1950; Adair 1934]. Every protein has an optimum pH, if the value is any higher or lower it is less effective until it reaches the point where it becomes denatured and no longer effective at all [Mitra et al. 2011]. At this point, the membrane no longer able to control ions that enter and exit the cells. The integrity of the cell membrane becomes compromised and substances from interior of the cell can escape, effectively killing the protein [Denice 2009]. In recent studies, it also discovered that the conformation of extracellular matrix proteins is also affected by changes in pH and temperature [Wu et al. 2009; Wu et al. 2007], denaturing the proteins and degrading their ability to bind cellular integrin receptors [Jones 2014]. Thus the acidity of the medium needs to be constantly balanced around pH 7 during the culturing period.

Additionally, the humidity around the cell culture chamber indirectly modulates the rate of evaporation from the culture medium with increased evaporation leading to a concentration of the salts in the medium.

Due to the sensitivity of the cells in the microenvironment, the culturing process must be carried out under strict laboratory conditions of asepsis, sterility and well controlled environment which can briefly summarised as temperature, humidity and gas (mainly oxygen and carbon dioxide) permeability [Abaci 2012], where those parameters, as stated in previous section, are reported to be crucial for maintaining a good viability and vitality of cells and thus facilitate the proliferation of cells in a governable manner [Brizzi 2012; Guilak 2009; Bissell 2002].



FIGURE 1.3: Illustration of Conventional Bioassay Techniques. Image from On-Line, refers to Website List

However, it is difficult to manage the consistency of microenvironments since the cell culture of bacterial and mammalian cells have mostly been carried out in conventional bioreactor devices such as bench scale bioreactors, culture flasks, culture-dishes and microplates which are illustrated in Figure 1.3. Those conventional cell culture methodologies are reported to have the following drawbacks: i) they are labour intensive and time consuming to operate, ii) susceptible to potential contamination from the large amount of human involvement, iii) a relatively large amount of metabolic waste is generated which can be toxic to the environment; iv) the culture environment fluctuates due

to the periodic replacement of culture medium; v) the time scale and process variables including temperature, pH and partial pressure of oxygen (p_{O_2}) are difficult to control and therefore the system often cannot provide a quantifiable culture environment, which causes difficulties and inaccuracies in further analysis. Further limitations include not being amenable to high-throughput screening, and conventional methods are often large-scale with high resource consumption [Abaci 2012].

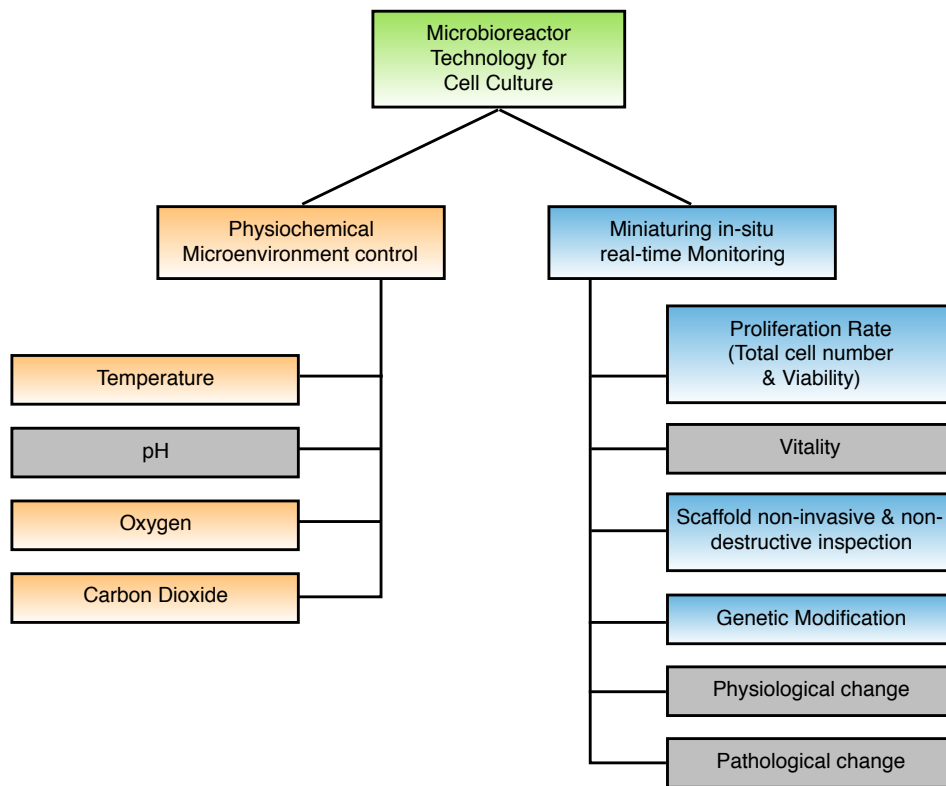


FIGURE 1.4: Illustration of microbioreactor technologies includes controlling parameters in physiochemical microenvironment and the potential indications of monitored cellular substrate via real-time monitoring system under the strictly controlled physiochemical environment

In response to the aforementioned challenges of controlling the microenvironment, there has been a drive during the last decade to develop microfluidic devices, commonly known as microbioreactors that operate in a well-controlled environment [Weibel 2006; Ruan 2009] (i.e. incubator, water-bath) at the time and length scales proportional to cellular phenomena [Liu 2007]. These microbioreactors are scaled down versions of conventional

bioreactors with low cost, high throughput screening [Ye 2007; Isett 2007]. The characteristics of microbioreactor are short transport distance and low volume requirements for cells, enabling cellular substrate to gain a fast response to surrounding microenvironment stimuli with reduced transport times of mass and heat which in a manner similar to the *in vivo* microenvironment [Taylor 2009; Ros 2008]. The microbioreactor allows microstructures that enable the generation of biological and physical gradients, which make it easier to study cells in modified microenvironments. In the past decade, there has been considerable effort in developing appropriate microbioreactors, based on different types of material, and platforms to quantitatively control the cell culturing parameters for cellular and tissue development.

Nevertheless, the published results also indicate that regardless of which type of cell is used, the biological cells characteristics often change in the culture process. Even small changes in temperature, pH, humidity and CO₂ levels could have an impact on a cells survival, growth, lineage of differentiation and metabolic state [Burdick and Vunjak-Novakovic 2009; Triaud 2003]. It is therefore possible that previous findings in a simulated environment may be different to those experienced in clinical cases, so there may be inherent risks if they are used in cell/tissue therapy [Dawson 2008; Atala 2004; Muschler 2004], hence there is strong demand for physiochemical microenvironment control in microbioreactor and the key parameters is illustrated in Figure 1.4.

Other than physiochemical microenvironment control, the cellular functional assay is also the key to succeed in cell culture. In general cell culture-based functional assays, the cellular response under various culture conditions are often studied through the investigations on cell counting and cell viability tests during the culture process to determine cell proliferation status [Ng 2005]. The viability results can be obtained through two approaches (i) quantification of cell counting, where fluorescent dye and fluorescent microscopic observations are heavily used [Oliver 1989]; (ii) evaluated through the indirect measurement of indicative metabolites that are released by the culturing

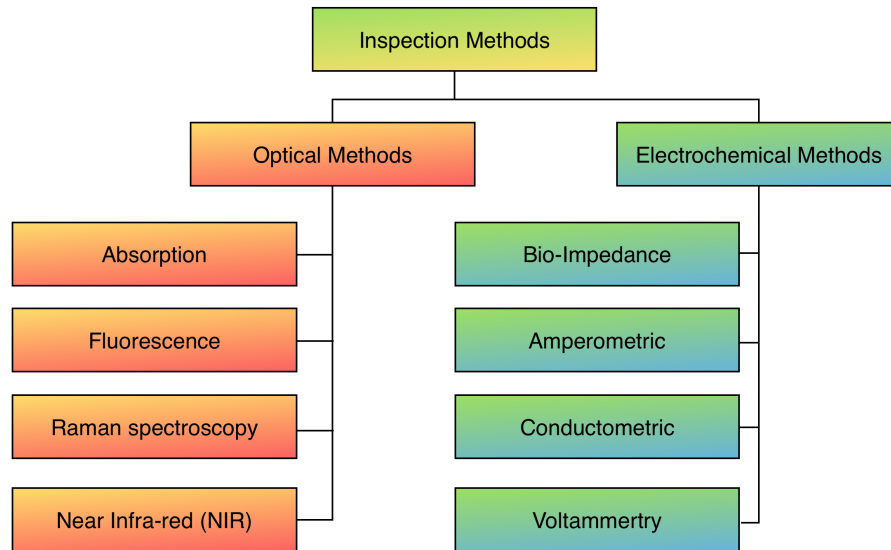


FIGURE 1.5: Summary of existing inspection methods for cell culture based functional assay

cells [Briggs 2009; Benes 2001]. Both of the above-mentioned conventional methods are time-consuming and require intensive labour operation during culture process and are difficult to implement in high-throughput screening applications [Ng 2005]. Hence, there is an urgent need for identifying, among the existing inspection methods, an alternative non-destructive and efficient assay, to quantitatively determine the cellular function and response in microbioreactor during cell culture. The existing methods are summarized in Figure 1.5.

A recent trend has been to move the monitoring and analysis closer to the customer [Ziegler 2000], such as in pregnancy tests and blood glucose concentration analysis for diabetes patients [Chu 2011; Wilkins 1996]. The trend of decentralization the monitoring system for biological substrate is expected to continue. Advances in both optical and electrochemical technologies have been enabled by analytical equipment, which has become portable, reliable and easy to use [Vyawahare 2010].

The development of microfabrication also facilitates the system to reproduce the same carefully designed biological experiments many times under similar specifications. Both developments have triggered the miniaturizing monitor system with rapid growing interest

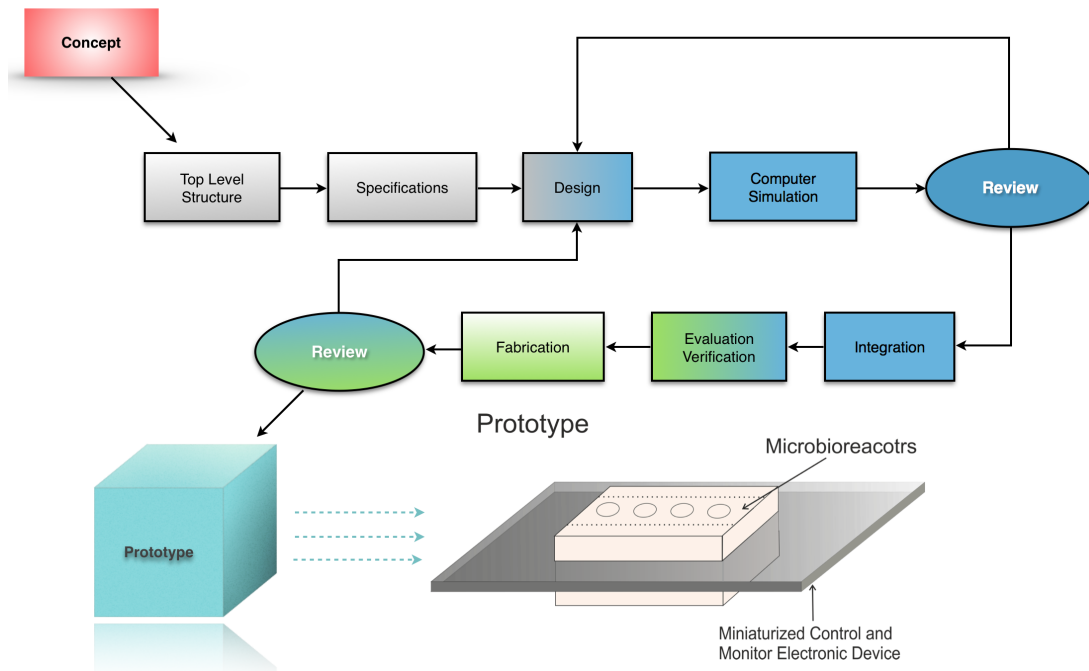


FIGURE 1.6: From concept to μ TAS. The successful development of a μ TAS involves a number of discrete steps includes identifying the specifications, design, simulate and evaluate performance, fabrication, and testing. Reviews of modelling and test results enable optimization of the performance of the μ TAS

in the development of Microsystems, defined as micro total-analysis systems (μ TAS) in 1990 [Manz 1990]. The concept of μ TAS builds on performing all the necessary steps that are required for biological monitoring and analysis on a miniaturized format and thereby offers portability, reliability, automation of optical and electrochemical analysis, high-throughput screening and real-time monitoring. The process of realization and developing a μ TAS system consists of several discrete steps as illustrated in Figure 1.6. The Concept refers to scientific significance and corresponding demand where the specifications depend mainly on the nature of electrochemical analysis as well as available existing technologies. It is often bounded by the type and desired range of detections, effect brought by physiochemical environmental changes and potential limitations. The fundamental theory of electrochemical analysis in turn determines the material and technologies that is suited to be utilised for fabrication of the μ TAS and a number of factors are included in the consideration such as reagents aggressiveness, optical transparency and inherent to combining mechanics, fluidics optics and electronics in μ TAS. With the

specifications in place, the next step is design, which constitutes the most important block in the flow diagram from μ TAS concept to prototype. It translates the scientific need and corresponding specifications into a fabrication plan. The following simulations are used to shorten the possibly long process of μ TAS design and hence optimise the miniaturising system efficiency.

In this thesis three separate systems were designed, integrated and fabricated into prototype where two of them focused on miniaturising the in-situ control and monitoring for cellular substrate in 3D cell culture in microbioreactor platform. In-parallel an automated system were also designed and developed to identifying the optical property of corneal collagen scaffold, which is expected to indirectly indicate scaffold applicability in Polydimethylsiloxane (PDMS) microbioreactor system.

1.2 Objectives of the Thesis

Based on the limitation of exist functional assay for cell culture, one may ask the question, is it feasible to fabricate a μ TAS system to be fully compatible with microfluidic devices that not only provide a homogeneous physiochemical microenvironment outside of conventional incubator but also be capable to implement a real-time in-situ monitoring, with excellent sensitivity and reliability, for cellular viability, growth rate (total cell number) and metabolic activity in both 2D and 3D cell culture format over a long period of time. By investigating, realizing and miniaturizing the existing novel technologies, including the physiochemical environmental control, optical and electrochemical monitoring elements, it ultimately integrates into a portable prototype for on-site analysis on PDMS microbioreactor platform (as illustrated in Figure 1.7).

The specific objectives are: (i) evaluate the hypothesis of an indirect, non-invasive thermal determination method on the combination of low thermal conductive PDMS

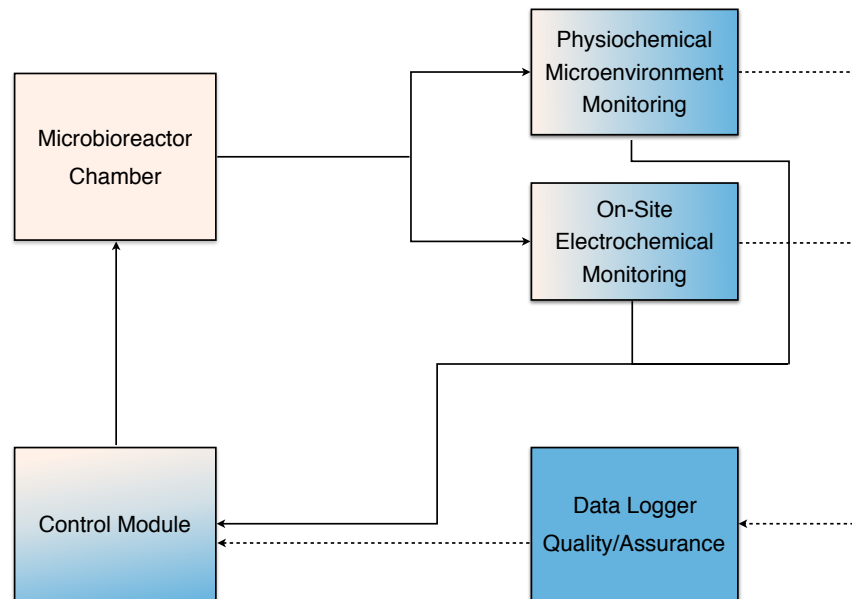


FIGURE 1.7: Functional block diagram for $\mu T A S$ that was proposed to provide a homogeneous physiochemical microenvironment outside of incubation area and is able to demonstrate the cellular viability and growth rate in both 2D and 3D culture environment without direct contact to cellular substrate

microbioreactor with utilised transparent heating element to control the microenvironmental thermal temperature and to assess the necessity of external supply oxygenate culture medium through microfluidic channels with a precisely controlled laminar flow rate; (ii) by employing the electrochemical cyclic voltammetry analysis, identifying the most suitable electrode material for long-term cell culture; (ii) determine the feasibility to construct an in-situ monitoring system by utilising the impedance analyser chip AD5933 with an multi-electrode switching matrix array and selected electrodes; (iii) validate the proposed alternative in-situ viability monitoring with conventional bioassays in 2D cell culture; (iv) identifying and evaluate a non-destructive and non-invasive inspection methodology for corneal collagen scaffold quality assessment with potentiality for high-throughput screening application; (v) utilizing the newly integrated in-situ assay method to quantitatively and qualitatively validate the inspected scaffold applicability for 3D cell culture and hence reveal the importance of microstructure of scaffold on the cell functions; (vi) in comparison to well established bioassay for metabolic activity

in 3D cell culture, identifying the sensitivity and reliability of integrated μ TAS system for time-lapse cell metabolic activity monitoring in 3D environment with designated scaffolds.

1.3 Structure of the Thesis

This thesis contains six chapters where the Chapter 1 provides a brief introduction to the research and an overview of the thesis followed with a few theoretical frame work that contains the general concepts required to understand the thesis. Chapter 2 focuses on the feasibility of utilising the Indium-Tin-Oxide film as the heating element to provide a microenvironment with improved thermal distribution in PDMS microbioreactor culture chamber. The concept of portability for microbioreactor with long term SH-SY5Y cell culture was also demonstrated in this Chapter 2. Chapter 3 probes the feasibility of using the Bioimpedance Spectroscopy (BIS), based on dielectric properties of cellular substrate, via Electrical Impedance Tomography (EIT) technology to provide an alternative and efficient method to monitoring cell viability in an a controlled environment outside of incubator. The integrability between miniaturised electrochemical monitoring methods and PDMS microbioreactor was also tested and verified. Chapter 4 investigates the concept of non-invasive and non-destructive inspection for type I collagen corneal scaffold, and demonstrates its biocompatibility with cellular substrate in microbioreactor. Chapter 5 discusses the reliability and adaptability of fabricated on-site analysing system to monitor the cellular culturing status, include viability and total cell number, for both 2D and 3D cell culture. The scaffolds were optically inspected via automated system proposed in Chapter 4 and were all categorized in the best quality region. The possibility of using Bioimpedance Spectroscopy to indicating the functional change for selected cell was investigated. Chapter 6 presents the general conclusions followed with an identified future work.

Chapter 2

Prototype Investigation of Indium Tin Oxide-Film based Portable Microbioreactor with Improved Thermal Distribution for Static Cell Culture

2.1 Introduction

For the proliferation purpose of biological cell culture, among almost all the published papers, both physical and chemical experimental parameters are constantly set to pH 7.4, 20 % oxygen in vapour space, 5 % CO₂ partial pressure and 37 °C for temperature [Laurent 2012; Haycock 2010; Keenan 2008] with desired humidity setting. Among those parameters, the humidity can be a vital factor to prevent the loss of culture medium via evaporation, especially in a non-sealed culture environment. The lack of permeability for oxygen and carbon dioxide will reduce the growth rate of cells to a great level [Wu 2006]. In addition to the humidity and gas permeability, the impact of temperature fluctuation has been studied during the past and a few studies suggest that the culture medium, which contains generally-used bicarbonate-carbon dioxide pH buffer, may become alkaline very quickly when cells are removed from the general lab-based bulk incubator and becomes harmful for cells. [Wink 1995; Owen 1991]

2.1.1 Physiochemical Factors

Generally, a small decrease in temperature from the optimum may slow down the cellular metabolism or cell growth rate, but an increase in temperature is likely to be harmful to the cells. It has been reported that the thermal environment in a static cell culture system could have a significant impact on the cellular physiology [Chong 2008; Brandam 2008; Peng 2007; Yoon 2007]. Moreover, the optimum temperature may vary between different types of cells. Research has shown that if the incubation temperature is set to 39 °C, the division process of hematopoietic cells is promoted [Bulter 2004], whereas an incubation temperature of 32 °C can reduce the damage from oxygen radicals to the mesenchymal cells and thus maintain the cells vitality and proliferation potential [Koobatian 2015 Stolzinger 2006]. Similarly, with other physiochemical factors, different types of cell or even the same cell in different proliferation stages would require different microenvironments. Thus, an adjustable heating micro environment is necessarily required under those conditions.

Other than temperature, pH control and manipulation of gas composition (e.g. O₂ and CO₂ level) is also important physiochemical factor for a microfluidic devices systems [Kostov 2001]. An optimum pH will enable high cell density growth and efficient enzymic activity [Maurer 2005]. If pH is not within the optimal range, the reaction rate could decrease dramatically. Previous studies show that, similarly to temperature, the optimum value for pH varies for different types of cells [Taylor 2009; John 2003]. A higher pH will favour the differentiation and maturation of megakaryocyte progenitor stem cells, whereas a lower optimum pH 7.1 will favour megakaryocyte progenitors [Schapper 2009; Betts 2005].

Oxygen has always been critical in highly metabolic stem cells, such as hepatocytes, culture processes and has often been taken into account for aerobic-fermentation microfluidic device design [Merkel 2000]. Particularly, due to the low solubility of oxygen

in aqueous solution (7.36 mg/mL at 25 °C in distilled water) [Grist 2010], both mammalian and microbial stem cells require constant replenishment of dissolved oxygen into the medium. If the oxygen is not provided appropriately, the metabolic rates and vasodilation in stem cells are reduced [Ozturk and Palsson 1991]. Research on static stem cell cultures have shown that the hypoxia environment not only slows down the stem cells differentiation process [Yushida 2009], but can also increase the stem cells proliferation during the culture. Under the circumstances where the oxygen partial pressure is maintained between 2 % - 5 %, the expansion of hESC and mESC proliferation rate was significantly faster than the rate of proliferation in 20 % oxygen partial pressure [Collins 2015]. Moreover, a humidified atmosphere is vital important to prevent the loss of culture medium by evaporation, particularly in non-sealed cell culture system [Hung et al. 2005; Giaccia 2004].

On the other hand, SH-SY5Y cells are a useful human cell line for reporting on the establishment of culture conditions. In their progenitor state they are robust and highly proliferative typically doubling in number every 24-48 hours. Following a 5 day exposure to high levels of Retinoic Acid (typically 50M concentration in basal medium), they differentiate into neuron-like cells that rapidly extend dendrites to form neural networks. Both the proliferation rate of the progenitor cells and the rate of dendrite extension of the differentiated neuron like-cells is sensitive to culture conditions and thus it is ideal to be used to validate the sustainability of the culture environment that is provided by the realized system.

2.1.2 Material and Fabrication of Microbioreactor

The previously mentioned sensitivity of cells during the culture process in bioreactor devices means that the control of physiochemical factors is crucial for maintaining the

consistency of culture conditions, such as temperature, pH and keeping survival and proliferation of cells in a governable manner.

As discussed in chapter one, in response to the aforementioned challenges in controlling the microenvironment, there has been a drive during the last decade to develop microfluidic devices, commonly known as microbioreactors that operate in a well-controlled environment [Young 2010; Shin 2008](i.e. incubator, waterbath) at the time and length scales proportionate with cellular phenomena and that are inexpensive to fabricate and ideal for high throughput screening [Vervliet-Scheebaum 2008]. The primary requirement of any material substrate used as a microbioreactor is the biocompatibility of the material so as not bring any negative effects to the adherence of the cells and cytotoxicity. The microbioreactor substrate must be chemically inert between itself and the cellular substrate without affect cellular growth factors [Barbulovic-Nad 2010; Van-Leeuwen 2009]. Furthermore, it should be sterilisable to eliminate bacteria, fungi and other material that may contaminate the cultured sample. The choice of the sterilisation procedure should not interfere with a materials optical properties [Lam 2004]. Other than biocompatibility of the material, the effectiveness of microbioreactor is achieved by considering a number of aspects into design and fabrication procedure including surface to volume ratios, mass and heat balance, and integrability with sensing and control elements. In this thesis, the concept of biocompatibility is referred to the ability of biomaterials, including polymers, electrodes and collagen scaffolds, to perform its desired function to generate the most appropriate beneficial cellular response and ultimately to accommodate and facilitate the long term 3D cell culture in microbioreactor, without introducing any undesirable local or systemic effects in the recipient or beneficiary to that cell culture.

Past researches demonstrated that a few material are suitable for fabrication of microbioreactors including polymers, ceramics, metals silicon, glass and wax. Polymers have

advantages over glass and silicon substrate in terms of cost and compatibility, but are often less ideal in terms of optical properties as compared with glass substrates [Patil 1988] and the difficulty of integration with other electronic circuitry [Strook 2002] in comparison with silicon [Becker 2000]. Among all the polymers reported, Wu et al.(2006) used the PDMS material for fabrication of microbioreactor due to its inherent nature which makes it suitable for cell-based systems and the ease of fabrication using prototype approaches [Wu 2008; Wu 2009]. The other unique technical advantages of the PDMS are summarised as follows: (i) high compatibility and flexibility to integrate with other system components and its non-toxic nature [Anderson 2000]; (ii) high permeability to O_2 and CO_2 [Bhattacharya 2005; Ng 2002]; (iii) a good transparency from UV to IR (230-1100 nm) regions of the electromagnetic spectrum, which enables the avoidance of potential interruption from added optical components during microscopic observation [Fleger 2006]. Furthermore, the PDMS surface can be easily modified by plasma

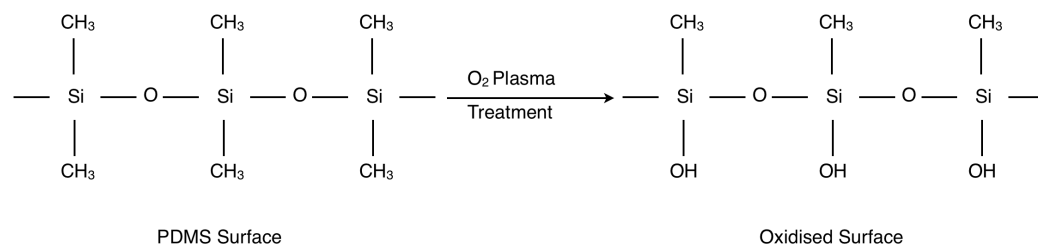


FIGURE 2.1: Surface Modification of PDMS by Oxygen-Plasma

treatment to make it hydrophilic (Figure 2.1). PDMS consists of repeating units of $OSi(CH_3)_2$ and, by exposing it to O_2 plasma, the CH_3 -groups on the PDMS-surface are removed and substituted by polar groups (OH) to create silanol ($Si-OH$) groups on the surfaces, thereby rendering them hydrophilic [Melin 2006]. The presence of the silanol ($Si-OH$) groups on the PDMS surface makes it more reactive to other silanes. The irreversible bonding will then occur between the PDMS-PDMS layers when the oxidised surfaces are brought into contact. The elastomeric nature of PDMS makes it ideal to integrate with micropumps, micromixers and microvalves [Eddings 2008 Jo 2000]. In

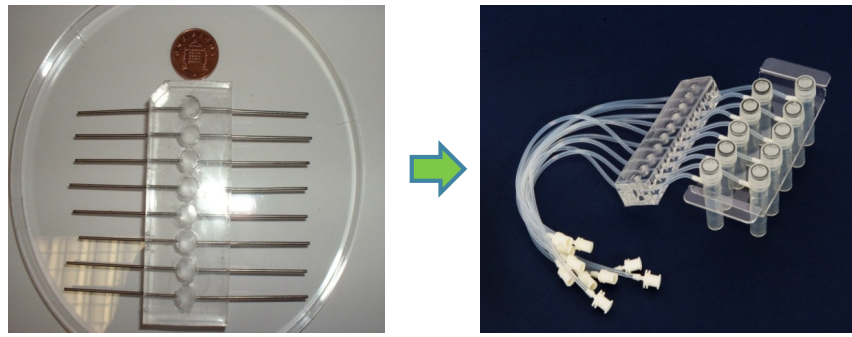


FIGURE 2.2: Demonstration of fabricated PDMS microbio reactor developed by MicroBio reactor-Technology Tissue Flex[®]

this DPhil study, the experiments used the PDMS based microbio reactor customised on the parallel perfusion microbio reactors platform, TissueFlex, which was developed by Cui et al (2007) which is shown in Figure 2.2.

2.1.3 Physiochemical Microenvironment Control

It is commonly known the physiochemical factors, includes temperature, pH, Redox potential, CO₂, O₂, are important for growth of cells and need to be precise controlled.

2.1.3.1 Oxygen Control

Oxygen availability to cells is equally important as temperature effect to culture process and it is essential to be addressed adequately in design and fabrication of microbio reactor that are based on aerobic fermentation [Chaturved 2013].

The availability of oxygen in conventional bioreactors systems can be increased by implementing a number of mechanisms such as perfusion with dissolved oxygen, surface aeration, bubble aeration and shaking [Betts et al. 2014; Huang 2007; Fleischaker 1981].

Membrane aeration is the most commonly used method for the supply of oxygen to microbioreactors (volumes 100-500 L) [Mehta et al. 2007]. Therefore, the oxygen demand from cells can be met by the diffusion of oxygen through a gas-permeable membrane such as those microbioreactors made from PDMS with diffusivity of oxygen $3.4 \times 10^{-5} \text{ cm}^2/\text{s}$ [Dendukur 2008]. Some studies in recent years have shown that oxygen supply can be realized without an external oxygen supply system [Huang 2013; Wu 2007] in PDMS by creating a large interfacial area via design principle for high surface to volume ratio. The oxygen needed for a single microbioreactor was estimated by assuming the concentration-independent oxygen consumption rate of chondrocytes is $2 \sim 4 \times 10^{-17} \text{ mole cell}^{-1} \text{ s}^{-1}$ and multiplying these values by the total cell numbers in a single microbioreactor. The oxygen supplied by diffusion through the PDMS layer can be estimated by Eqn. 2.1.

$$F_{max} = D_{PDMS} \bullet (\Delta C / \Delta Z) \quad (2.1)$$

where ΔZ represents thickness of the PDMS layer, and ΔC represents oxygen concentration difference across the PDMS layer and D_{PDMS} denotes the diffusivity of oxygen in a PDMS polymer. Under this circumstances the D_{PDMS} was estimated to be $4.1 \times 10^{-5} \text{ cm}^2/\text{s}$ [Charati and Stern 1998] and $\Delta Z = 2 \text{ mm}$ (thickness of PDMS slab). The oxygen concentration at the interface with atmosphere is $2.0 \times 10^{-7} \text{ mole } O_2/\text{cm}^3$ and, assuming oxygen concentration at the fluid outlet equals to zero and all oxygen is consumed by cells, ΔC is $2.0 \times 10^{-7} \text{ mole } O_2/\text{cm}^3$. F_{max} can then be estimated as $1.1 \times 10^{-7} \text{ mole } O_2/\text{reactor} \cdot \text{day}$, which was reportedly at least an order higher than chondrocyte oxygen consumption.

However, ITO film (with 0.34 mm thickness) is known to be a gas barrier and there is negligible mole flux diffuse through the film [Ohishi 2003; Henry 2000]. Thus, by adding the ITO film on the bottom side of PDMS microbioreactor, as shown in Figure 2.3,

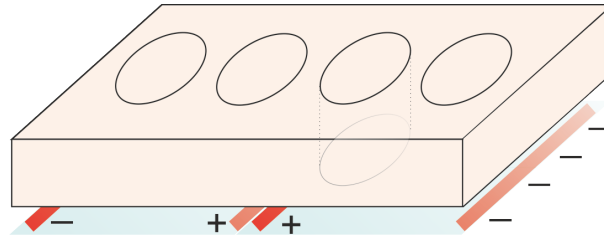


FIGURE 2.3: Illustration of Heating System with ITO-film that firmly attached to the bottom of PDMS microbioreactor

fewer gases will diffuse into the well and only through the top side surface of the PDMS. An external oxygenate culture medium may be necessary through microfluidic channels with a precisely controlled laminar flow rate. The necessity of external laminar flow is assessed indirectly by determining proliferation growth rate under different conditions and was discussed in the later part of this chapter.

2.1.3.2 Thermal Control

One of most important physiochemical factors is considered to be temperature since the rate of cell doubling and enzymic processes are both dependent on temperature. However, the control for temperature is reported to be a challenging task due to the high rates of heat transfer of cell culture platform, that can gain and lose heat very quickly. This heat transfer rate is often affected by the surface area to volume ratio of the bioreactor as well as the heating element position in the system; both factors generally cause the temperature to fluctuate during the culture process. Hence there is a clear demand for a precise thermal control system; it is critical to find a heating material and its position that does not create high temperature gradients inside the culture chamber.

Schapper et al (2009) and Geschke et al (2008) have thoroughly reviewed the temperature and pH effect to microbioreactors, where thermistors and resistance temperature

detectors (RTD) were used as the thermal sensor for thermal control. Three methods exist in the literature to control the temperature of microbioreactor systems: (i) a heating element based system; (ii) programmable temperature controlled incubators; (iii) controlled water baths. One of the major challenges of method (i) is the high rate of heat transfer to and from the microbioreactor, which causes abrupt fluctuations in temperature. Because of this, methods (ii) and (iii), known as conventional cell culture heating system, are often carried out in carefully calibrated and monitored lab-scale incubators where the temperatures are set to be 37 °C, and are often based on the principles that the chamber temperature is effectively controlled by a large jacket through which the circulation of water can help to evenly distribute the temperature field with a fluctuation of 0.2 °C. The temperature is precisely controlled either in the incubation or in the water bath by directly sensing the temperature in the macro-environment. By introducing the microbioreactor into the system, the desired temperature inside the bioreactor is obtained through the thermal balance between the macro-environment and the microenvironment. The practical type of incubator varies from a temperature-regulated box to a more complex system which can monitor and control temperature, humidity and oxygen.

Although the fluctuation of temperature tends to be small in a well-controlled incubator / water–bath environment, these systems are commonly bulky and not well compatible with other cell culture experimental setups since the interconnection of the medium feeding tubing with external medium pumping equipment is normally required. Furthermore, the control system uses a direct sensing system, making it difficult to conduct parallel experiments under different thermal conditions. There is a strong demand and need for a homogenous and customisable environment outside of the incubator for cell culture for microscopic observation and other online monitoring activities; the conventional incubator setup complicates these experimental operations.

2.1.3.3 Heating Element

Few studies examined semiconductor light sources (light emitting diodes, LEDs), with seven different wavelengths, to illuminate and heat the wells during the cell culture process. However, these materials only convert 20 % - 30 % of power into visible light [Uhl 2006]; this low power efficiency is a major obstacle for portability. Other unconventional materials have been used as heating elements in recent years, such as light-weighted peltier semiconductors; this is composed of a large number of heavily doped p-n type junctions in series connection. This system has both reliable heat efficiency and an improved thermal distribution; furthermore, it has the unique advantage of excellent system integration as the top plate can be used to heat the microbioreactor during the cell culture, while the bottom cold plate can be used to cool the electronic microprocessor and motherboard. However, peltier semiconductors consume significant power and is not transparent, making it unsuitable for microscopic observation during culture. The

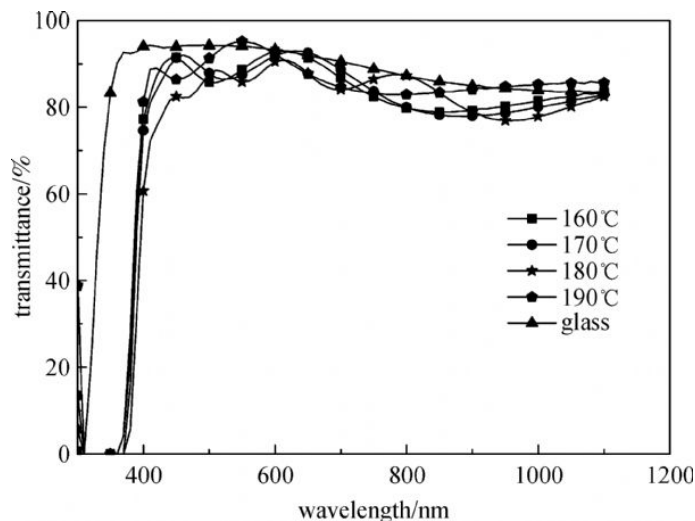


FIGURE 2.4: Transmittance rate of a typical ITO film with thickness of 1 mm under different thermal conditions [Zheng et al. 2013]

emerging material that is gaining popularity as a heating element is Indium Tin Oxide. The integration of the ITO as the heating element is a relatively new concept, where the ITO is a heavily-doped n-type semiconductor. It is extensively used as a transparent and colourless thin layer in optical coating. The ITO can be deposited on glass and

polymer substrates. Literature suggested the conductivity of ITO glass is better than ITO film [Li et al. 2015; Shigesato 2000], nevertheless the coating of ITO film is more durable and is not liable to cosmetic damage such as oxidation from moisture like a silver based coating on ITO glass.

In principle, as a heavily-doped n-type semiconductor, by applying a voltage difference on the side of ITO film, it increases the number of free moving charges on the film [Hong et al. 2010]. As a result, the current in the conductor is due to the drifting of electrons inside a conductor in a direction opposite to the flow of electrons. During electrons drifting they collide with their atoms vibrating about their mean position and lose some of kinetic energy to the atoms which ultimately increases the amplitude of those atoms' thermal energy. Hence the temperature of the conducting material rises correspondingly. Furthermore, ITO-film is also a good compromise amongst all other transparent conducting films; it is thick enough to carry a sufficient current but thin enough to be transparent [Mohamed 2016; Granqvist 2002]. A further five reasons to choose the ITO film as the system heating element are: (i) anti-moisture [Bashar 1998]; (ii) low resistivity; (iii) it allows 80% transmittance in the visible region [Zheng et al. 2013] as shown in Figure 2.4; (iv) inexpensive; (v) low temperature coefficient of resistance since the data showed the heat test ratio (R/R_o) remains at 1.03 for a continuous heating of 150 °C for 30 mins. The low resistance implies low power consumption for the heating process and it also helps in terms of system output control, this means the power required for the heating unit will remain nearly constant as the temperature rises in the microenvironment.

Past research shows that very limited discussions about the thermal distribution of a larger area of ITO-film based heating zone to be used in biological researching field. In terms of cell physiology, the feasibility of exploiting the optimized ITO-film as a heating element in an indirect and non-invasive thermal control system in a static cell culture, outside of a cell incubator, has not yet been fully explored. Thus, the goal is to develop a

labour-free, portable, optically transparent, energy efficient lab-on-chip monitoring and control system for cell culture in a microbioreactor, which is outside the incubator and for experiments under different temperature operating conditions.

During the past years, a pioneering part of the overall project has been implemented and primarily focused on a prototype for indirect temperature control of a bioreactor by utilizing the innovative heating element, Indium-Tin-Oxide. Particularly, Lin et al.(2007) initialized the development of ITO glass based micrometer chip for situ fluidic heating and thermal control in a microchip for cell immobilization applications. A similar chip was realized by Cheng et al.(2008) with thermal stability and intended to utilize the system for long term cell culture. Both of the above-mentioned devices rely on the fine coating of ITO on the surface of the glass substrate. However the fine coating of ITO on the surface of glass substrate is achievable only through industrial processes and lack of user-end customisation. The reported excellent thermal distribution and precise temperature control demonstrated in the literature [Lin et al. 2011] is mainly referred to the ITO glasses rather than ITO polymer film and is only focus on small heating area ($5\text{ mm} \times 5\text{ mm}$) of patterned zone. Thus there is a need to investigate the feasibility of using ITO-film as a heating element for larger areas with improved thermal distribution.

The limitations of ITO film were addressed in the past literature. As a transparent conductive heating element, increasing the thickness and increasing the concentration of charge carriers will increase the material's conductivity, but decrease its transparency [Zhang 2001; Alam 2000]. The non-uniformity of the generated heat which may cause abrupt fluctuations in temperature due to the high rates of heat transfer to and from the microbioreactor [Caminoa 2001] was the one of issues dissuaded from other papers [Betz 2006; Guillen 2005; Granqvist 2002]. Further details including the transmission characteristics over microscopic observation have been discussed in the method and experiment sections.

The two challenges in this project are (i) finding an optimized solution to generate a thermal distribution without introducing high temperature gradients, since it directly affects the efficiency of temperature control in microbioreactors, and (ii) assessing the feasibility of a non-invasive and indirect detection method to monitor and control the temperature in microbioreactors, where each micro-chamber contains a low thermal conductivity and a relatively high value of specific heat capacity [Frenkel 2016; Chen 2005] (a high value of specific heat capacity and low thermal conductivity means a longer time for the system to reach equilibrium which causes a thermal transfer delay and is regarded as a non-linear process variable). The system should use reliable, cheap and scalable feedback thermal-sensors to fulfil these requirements. The indirect sensing feedback is essential as direct ceramic-based thermal detection methods are prone to contaminating the cell culture.

Due to the characteristics of low thermal conductivity for PDMS, a simple proportional (P) integrated (I) derivative (D) feedback control (known as PID) algorithm is recommended for use. In principle, PID control is a control process to operate proportional, integral and differentiation for error e , which is defined as the outcome difference between the set-point (SP) and process variable (PV). The combination of P, I and D parameters will determine the next operation that the system should deploy. Among those three factors, the differentiation operation has strong predictability and also speeds up the process, so a PD process is often chosen as a control algorithm for temperature. Nevertheless differentiation relies on precise measured feedback which, by considering an indirect measuring system with low thermal conductivity physical property, is hard to obtain.

In this chapter, we discuss the design principle and implementation of a labour free energy efficient, indirect detection-based temperature monitoring and controlling system based on cheap and scalable components. A Stein-Hart equation, as a model of the resistance of a semiconductor at different temperatures, was used to prove the reliability

of cheap and scalable components compared with a scientifically proven accurate thermal sensor, 10k TH thermistor. Furthermore, the heating element, Indium Tin Oxide (ITO) Film, which is used to supply heat to Polydimethylsiloxane (PDMS) for both 2D and 3D cell cultures, its electric and optical properties were investigated. ITO films of differing structure were also analysed in order to obtain an optimal thermal distribution. This was achieved through the thermal energy distribution simulation via Energy2D software and the concept is demonstrated by a large number of experiments using Infra-red thermography. The indirect method of sensing, as feedback in the system, for measuring and controlling temperature was a proposed hypothesis and the final experimental results show its effectiveness (with the help of a basic PID algorithm). A microprocessor and switching-relay based system combined with a reliable data collection unit for controlling temperature in a microbioreactor was designed and assessed both individually and in the system. The switching system is flexible allowing for integration with other control components in the future and consumes little power. The overall results on a GUI interface show a reasonable capability of control at 37 °C in a microbioreactor via an indirect sensing method. Finally, human neuroblastoma cells (SH-SY5Y) were cultured in the microbioreactors and cell morphology and viability were assessed. The thermal environment generated by the ITO heating element was found to facilitate SH-SY5Y cell culture comparable with use of a conventional cell incubator. In summary the demonstrated results facilitate the concept of portability for a microbioreactor which can be regarded as a positive movement towards a totally controlled and real-time monitored cell culture system.

2.2 Material and Method

2.2.1 Fabrication of Microbioreactor

Since the material of the bioreactor is a kind of silicone elastomer, the microfluidic structure is fabricated by replica molding processes with a mold master (made of photolithography of SU-8 2100 negative photoresist on a silicon wafer), which contains the negative pattern of the structures with desired the size and the shape of the well. In this case, the circular based cylinder well has a diameter of 6mm and depth of 6mm and the desired dimensions for the bioreactor are $100\text{ mm} \times 29\text{ mm} \times 13\text{ mm}$. A complete mechanical mixture of liquid-state pre-polymer PDMS with its curing agent is required in a ratio of 10:0.9. The mixed liquid-state PDMS is then poured on the master for fabrication of both cover and chamber unit, meanwhile four thermistors were introduced into the liquid-state PDMS for the bottom side of the bioreactor. The polymer with thermistor was then de-aerated under vacuum to remove all air bubbles created during mixing followed with solidification process. After baking for solidification, both PDMS layers are peeled off from the master. Inlets and outlets of each PDMS layer are drilled with 1 mm diameter and three thermistors are solidly positioned with a horizontal distance of 1.5mm to the culture chamber and one thermistor is semi-embedded for exposure to the surrounding air. Moreover, non-toxic and non-corrosive fluid conduits (stainless steel-based rigid tube) are provided in each well, as inlets and outlets with 1 mm diameter. This enables the supply of culture medium and removal of metabolic waste as well as the spent medium from the cell culture via the insertion of biomedical-use needles into each reaction well directly through the side wall of each well. However these fluid conduits have mainly been used for other purposes in the experiments which will discussed in a later section.

2.2.2 Preparation and Investigations of Heating Element

An automated and portable ITO film-based heat control device, with a large surface area of heating zone for cell culture outside of incubator, was realized and tested. Conventional lab-manufacturing methods for the ITO-based heating element use patterning ITO on a substrate via wet etching techniques [Lin et al. 2007] and directly screen print electrodes on the surface of ITO [Lin et al. 2010]. I used a commercially available and low cost ITO film from Panel Graphic Ltd, with dimension of $1.6\text{ m} \times 0.48\text{ m} \times 0.34\text{ mm}$, as the heating element in this experiment. This commercialized ITO film has an added optical clear adhesive layer attached to the top of the film. The film was immersed in a solvent cleaner in order to remove any surface contamination which may prevent adequate material contact. A two parts silver vial epoxy adhesive material (10 g, RS Ltd) was prepared by mixing equally weighted 1.5 grams from each parts of adhesive material thoroughly for 3 minutes. The mixture was later added between two copper electrodes and conducting layer of the dried ITO film. The bond was cured at 80°C for 15 minutes in order to maximise the conductivity and adhesion. A further enhancement in contact was obtained by adding copper tape at the contact junction. Thus the electric conductive bonding was facilitated and ultimately improved the power efficiency for the ITO-film based microheater system. The photographs of the front and rear side of the fabricated ITO-film based heating element are shown in Figure 2.5.

The temperature dependence of electrical resistance for heating element and thus of electronic devices has to be taken into account when constructing devices and circuits. The coefficient, α , is thus critical as it describes the relative change of a physical property of the ITO film that is associated with a given change in temperature and can be mathematically expressed as

$$\frac{dR}{R} = \alpha dT \quad (2.2)$$

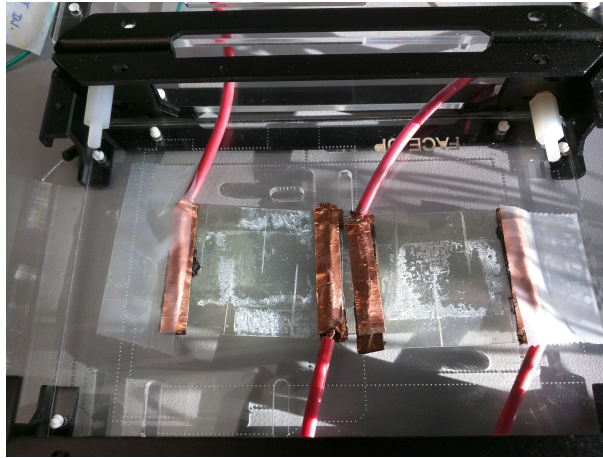


FIGURE 2.5: Illustration of fabricated ITO-film heating element

α has the dimension of an inverse temperature and can be expressed in K^{-1} . The temperature range was selected from 24 °C to 44 °C based on the need of thermal environment for cell culture. Experimentally, temperature coefficient was determined by repeatedly placing an ITO film in a uniformly thermal distributed environment where the temperature was precisely controlled with incrementally increase. A further power consumption analysis was implemented by directly exerting the ITO film (100 mm $\times$ 29 mm) via copper electrodes, which shared the same surface area with the fabricated PDMS microbioreactor. Other than the electric properties of ITO-film, an optical transmittance evaluation was also implemented based on microscopic recording for static SH-SY5Y cell culture experiment in PDMS microbioreactor on day 3 with a specifically selected wavelength range including Green (495-570 nm) and Red (620-750 nm).

2.2.3 Evaluation of Thermal Distribution

Both literature and point-based electric experiments indicate the lack of uniform thermal distribution on the ITO-film with a large surface area. It is thus necessary to investigate the distribution of the temperature field on the ITO-film surface. Due to the fact that only static cell culture was implemented, it is acceptable to assume that the heat transfer was dominated by heat conduction. Hence heat radiation was neglected and heat

conduction generated from ITO film conductive surface, which directly attached to solid PDMS chamber, was considered. Here a 2-D heat conduction model for heterogeneous media is modelled using the following partial differential equation:

$$\rho c \left(\frac{\partial T}{\partial t} + \nabla [vT] \right) = \nabla [kT] + q \quad (2.3)$$

where k is the thermal conductivity, c is the specific heat capacity, ρ is the density, v is the velocity field, and q is the internal heat generation. The heat equation has two parts: diffusion part characterised by the conductivity field function and the advection part characterised by the velocity field function. The rate of heat flow from one side of ITO-film to the other is one of the major factors for the surface conduction process, since copper electrodes act as both a power-based heating source and heat sink (the inhomogeneous case). The Fourier law of heat conduction was included in the consideration and Eqn. 2.3 it can be mathematically simplified as

$$q_{ht} = -kA \frac{\partial T}{\partial x} \quad (2.4)$$

where the q_{ht} stands for heat transfer rate of the source in watts, T is the temperature and k is the thermal conductivity as stated previously, A is the cross sectional area of ITO-film heating path and $\frac{\partial T}{\partial x}$ is the temperature gradient in the direction of the flow (K/m). Geometrically, the Eqn. 2.4 can be further expressed as

$$q_{ht} = -kA \frac{\Delta T}{L} \quad (2.5)$$

where L is the semiconductor ITO-film length, T represents the simultaneous temperature difference two electrodes and the expression $\frac{\Delta T}{L}$ is known as the temperature gradient and is vectorially represented by the quantitative changes in temperature per unit of distance moved along the path of heat flow on ITO-film surface. The heat flow per unit area, known as heat flux along the pathway through ITO film, q''_{ht} , is then

defined as

$$q_{ht}'' = -k \frac{\Delta T}{L} \quad (2.6)$$

The side of ITO film, which is excited by voltage, is considered as heat source and its generally assumed that the proportion of absorption (absorptivity) is 80 %.

$$E_{in}(absorbed) = E_{in} \times 0.8 \quad (2.7)$$

E_{in} can be found by dividing cross sectional area of transmitted pathway on ITO film into power supply over times (P/A). Moreover, from energy balance perspective, once the surface of ITO film reached a steady temperature

$$E_{in}(absorbed) = q_{ht}'' + E_{out} \quad (2.8)$$

It is part of the assumption that heat convection does not take place during the equilibrium process. E_{out} , as radiated energy, can be expressed by using Stefan-Boltzmann equation

$$E_{out} = \epsilon \sigma T^4 \quad (2.9)$$

Where ϵ represents emissivity of ITO film, σ is the Stefan–Boltzmann constant (5.67×10^{-8}) and T is absolute temperature in Kelvin. The emissivity of ITO surface was also found to be 0.8 from the material data sheet provided by the manufacturer. By substituting the Equ 2.6 into Equ 2.8, the heat flux travel distance as equilibrium condition for ITO film's temperature reaching its steady state is then expressed as

$$L = \frac{\epsilon \sigma T^4 - (P/A) \times 0.8}{-k \Delta T} \quad (2.10)$$

The ΔT was set to be 0.5 ° C and overall length of the charge carrier's pathway on ITO film for reaching heating equilibrium conditions was found to be 93.78 mm. To verify the measured steady-state temperature contours, a conduction simulation was completed

Material	Conductivity: k ($Wm^{-1}K^{-1}$)	Density: ρ (kgm^{-3})	Specific Heat: C ($Jkg^{-1}K^{-1}$)
ITO film	2.34	1380	1000
PDMS	0.15	970	1460
Water	0.61	997	4160

TABLE 2.1: Material properties of ITO-film, PDMS and water at the conditions of temperature: 298 K and pressure: 1atm

using Energy2D software. Free convection and adiabatic condition were imposed on the surfaces of the microbio reactor chamber. A constant heat generation rate was assumed to apply on the ITO-film based heating element before reaching its steady state. The numerical geometry was mainly constructed by three parts including a) PDMS microbio reactor cover, b) microbio reactor chamber and wall, c) PDMS membrane and d) ITO film. The numerical dimension of a single microbio reactor chamber is 15.58 mm diameter with 10.58 mm depth. The non-uniform mesh grid was used for these numerical simulations to help illustrate the simulation results along the vertical axis for a single culture chamber. A few critical parameters required for the simulation are listed in Table 2.1.

The thermal distribution of the modified conductive surface of ITO film and proposed microheater system was validated via infrared (IR) thermography (FLIR i7, Monition UK) with and without the PDMS layer. In this work, the focal plane was perpendicular to the detective light and the measurement distance to the focal target was set to be 30 cm. Before the observation, the accuracy of the temperature measurement was checked to be $\pm 1^{\circ}C$. The emissivity for IR imaging was set as 0.88, 0.92 for the material of PDMS, ITO film respectively.

2.2.4 Design Principle

To realize the proposed ITO-film based heating system, a micro-processor (Arduino Uno3 Italy) with a 10-bit analog-digital converter and 8-bit pulse-width-modulator (PWM) was utilized. The maintenance for temperature in the culture chamber, with minor fluctuations, was achieved by utilizing the simplified PID as the control loop feedback mechanism in the designated temperature control system. Briefly, the validated thermistor served as a sensor to monitor the local temperature condition and indirectly indicated the thermal environment inside of the targeted culture chamber. The generated signal was sent to the microprocessor module, which output an electric current and thus the corresponding temperature condition on the ITO-film based heating system was constantly modulated. The Figure 2.6 (a) illustrated the top-level design and (b) shows the ITO-film based portable heating device.

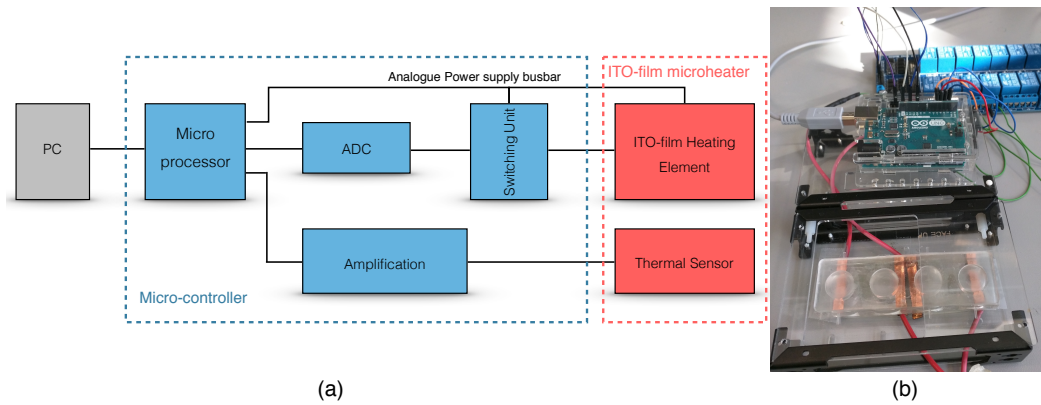


FIGURE 2.6: (a) Schematics diagram of the feedback thermal control loop, (b) A photograph of the hand-held micro-controller with an ITO-film microheater chip connected

2.2.5 Thermal Sensing Element Characterisation

To achieve high precision data recording for temperature measurement in the ITO–film based heating system, it was necessary to characterize the available thermal sensing element to identify the thermistor that has similar physical properties to a well-established thermocouple in industry with excellent sensitivity. A micro-temperature controller, mTCII-HT100, was initially introduced in 2013 and the system has been reported with exceptional accuracy for temperature detection by employing a high precision thermistor, TH-10Kmp, with standard error of ± 0.2 °C. The Steinhart-hart model which is known as a model of the resistance of a semiconductor at different temperatures was utilized during the characterization process.

$$\frac{1}{T} = A + B \ln(R) + C (\ln(R))^3 \quad (2.11)$$

where T is the temperature, R is the resistance at T (in ohms) and parameters A , B , C are determined based on the measured data. The closer the range of temperature and corresponding resistance is applied to the model, the more accurate an expression can be obtained. Since the thermal sensors did not heavily fluctuate within the selected temperature range, the temperature coefficient and the electrical property of the each sensor, associated with the given temperature, was identified using a linear regression of the Steinhart-hart model.

$$R(T) = R_0(T_0)(1 + \alpha \Delta T) \quad (2.12)$$

The value R_0 represents the resistive characteristics of the sensor at a reference temperature T_0 and ΔT is the difference between selected temperature, T , and reference temperature, T_0 .

2.2.6 Investigation of Non-Invasive and Indirect Thermal Detection

The literature suggests that PDMS has low thermal conductivity and high volumetric heat capacity and, as a result, it takes longer for the system to reach equilibrium, causing a thermal transfer delay [Frenkel 2016; Chen 2005]. This effect is known as thermal inertia and is a non-linear process. Thus, a steady-state calculation can yield inaccurate results. The thermal inertia of the surface is determined by small-scale features of that surface. A number of thermal tests for the microbio reactor system were implemented by deploying the selected thermistors at various positions within the heating system that contained the material with high thermal inertia property. The Steinhart-Hart model was used to demonstrate the concept and feasibility of this non-invasive and indirect thermal monitoring system. The results also contributed in determining the parameters in the flow of control. The setup of the experiment is illustrated in the Figure 2.7. The

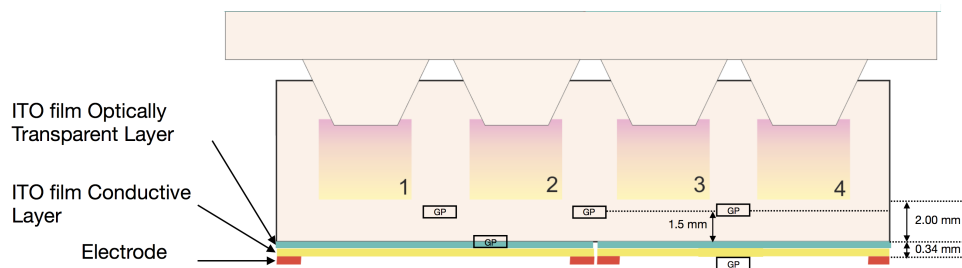


FIGURE 2.7: Side View of Microbioreactor Indirect Thermal Measurement Setup. Three pre-embedded GP thermistors were positioned inside of the microbioreactor with a horizontal distance of 1.5mm from culture chamber 1, 2 and 3 respectively and 1.5 mm distance from ITO film conductive surface. Two additional GP thermistors, one under culture chamber 2 was semi-embedded to touch the ITO-film transparent layer and another sensor is fully attached on the conductive surface of ITO-film.

mTCII with TH-10Kmp sensor was introduced to measure the temperature inside of the chamber with and without aqueous solutions (culture medium) simultaneously by inserting the sensor into the air chambers through the stainless still well conduits. Two ITO films ($35\text{ mm} \times 29\text{ mm}$) in this system are connected in parallel and it is their optical transparent layers that are attached to the PDMS bioreactor via araldite agent.

A damping factor was identified in the multi-cyclic experiment and a new correlation was added to the control feedback algorithm.

2.2.7 Switching Unit and Development of Graphic User Interface

In addition to the characterization of ITO-film, an evaluation was also made for the capability of micro-processor to supply the necessary power required by the heating element over long term cell culture. A pair of switching relays was controlled by a microprocessor which communicates with the PC and each relay is assigned to a single ITO-film based heating element. A standard GUI interface enabled the PC to communicate with the switching unit via the Arduino Uno board in an I²C protocol format. The time interval range for feedback data collection was set by the GUI interface, where the temperature conversion is calculated by the new correlation obtained from the thermal test for the bioreactor system section; the system allows upper and lower temperature limits to be manually re-defined. An export to excel function has also been developed which enabled further analysis for transient response.

2.2.8 Cell Maintenance for SH-SY5Y

Human neuroblastoma cells (SH-SY5Y) originally purchased from ATCC (LG Standards, UK) and transduced to stably express mCherry fluorescent protein by David Nagel (Aston University, UK) were a gift from Eric Hill (Aston University, UK). They were cultured in 25 cm² flasks in 5 ml of complete medium consisting of RPMI with added L-glutamine (Sigma-Aldrich, UK) supplemented with 10 %(v/v) Fetal Bovine Serum (FBS, Gibco, ThermoFisher, UK), 1 %(v/v) MEM-Non-Essential Amino Acids Solution (100 ×) (ThermoFisher, UK), and 1 %(v/v) Penicillin (1000 IU/mL) / Streptomycin (100 ug/ml) (ThermoFisher, UK). Cells were maintained in an incubator at

the temperature of 37 °C in a humidified atmosphere consisting 5 % CO₂ and 20 % oxygen in circulated airflow, and medium was changed every 2-3 days. Prior to cell seeding, ECM-Gel from Engelbreth-Holm-Swarm murine sarcoma (Sigma-Aldrich, UK) was evenly coated across the bottom of each of the PDMS wells (60 *l*/well) to create a cell attachment layer on the bottom surface of the chambers, and chambers were sealed using a PDMS lid and incubated at 37 °C to set the gels. Cells were split and used when confluence reached 70-75 % by removal of the cell culture medium, washing in 5 *mL* of PBS and harvested following a 3 *minute* incubation in 1 *mL* of trypsin-EDTA solution. To neutralise and stop the action of trypsin-EDTA, 5 *ml* of fresh complete medium was added to the flask. The 8 *mL* of the solution was then pipetted up and down several times to obtain a homogenous distribution of cells in solution. Cell count and viability was measured using a hemocytometer. The solutions were then centrifuged at 800 *rpm* for 5 *minutes* and the supernatant was disposed, leaving a pellet of cells (1.4×10^5) at the bottom of the centrifuge tube. This cell pellet was re-suspended in complete culture medium to obtain the desired cell seeding density (5×10^3 *cells/cm*²). Cells were seeded into each chamber in three different sets of PDMS microbioreactors and cultured using 300 μ *l* of complete medium per well. Unused cells were incubated and passaged in culture flasks for future experiments.

2.2.9 Bioassay

SH-SY5Y cells, previously transduced to express mCherry fluorescent red protein, were imaged using reflected epifluorescence light (568 *nm* excitation / 620 *nm* emission wavelengths) using an inverted Nikon TIE microscope with a 10 $\times$ objective, a cool LED illumination system and an EXI Blue cooled CCD camera. Cells that were found to express mCherry protein were clearly visible using relatively short exposure times (≤ 1 second) and counted as viable. For each chamber, at least 5 fields of view were imaged

and the number of cells averaged and extrapolated against the visible area to obtain a total number of cells per well under three different thermal conditions.

2.2.10 Data Analysis

Data here are presented as the mean $\pm$ the standard deviation of the mean of triplicate experiments. The analysis of ITO-film optical property in transmittance under conduction circumstances were implemented via a two image processing algorithm, consisting of Peak Signal-to-Noise Ratio (PSNR) and Structural Similarity Index (SSIM) . The PSNR was obtained by implementing the following equations in the algorithm:

$$PSNR = 10\log_{10}(MAX^2/MSE) \quad (2.13)$$

where MAX is maximum possible pixel value of image datatype and given a noise-free $m \times n$ monochrome image I and its noisy approximation K , the MSE is defined as

$$MSE = \frac{1}{mn} \sum_{i=1}^{m-1} \sum_{j=1}^{n-1} [I(i, j) - k(i, j)]^2 \quad (2.14)$$

m and n are the number of pixel in row and column of a image. The algorithm of SSIM can be found in Appendix A. Data are presented as the mean $\pm$ the standard deviation of the mean of triplicate experiments. For the comparison of total cell number after two days static cell culture, Student's t-test with a statistical significant level of 0.05 was used.

2.3 Results and Discussions

2.3.1 ITO-film Characterisation

Indium tin oxide film is one of the most widely used transparent conducting material. However, a compromise must be made between conductivity and transparency, since increasing the thickness and increasing the concentration of charge carriers will increase the material's conductivity, but decrease its transparency. Thus it is always necessary to characterize the ITO-film with regards to its electric and optical properties.

2.3.1.1 Electric Properties of ITO-film

Both electric and optical properties of ITO-film under conduction circumstances were analysed. The temperature coefficient at each specifically given temperature was found via linear approximation; the values are displayed in Table 2.2. The average resistance of the ITO-film remains in a range between $20.6\ \Omega$ to $20.9\ \Omega$ as temperature varies from 25°C (room temperature) to 40°C , and the ratio between the most out of set value to the mean value is 1.03. The low resistance value implies low power consumption for heating process and the insignificant variation also favours the system output control. Meanwhile the temperature coefficient of ITO film within this temperature range were all insignificant. The temperature coefficient was often employed to expressing the relation between a change in electric property (resistance) and the change in temperature that causes it. The obtained results revealed an excellent consistency of ITO electric property over designated temperature range.

The silver loaded epoxy adhesive material was utilized with conductive copper tape to improve the contact between electrode and conductive layer of ITO-film, thereby reducing the systematic errors in identifying its electric properties (in terms of power consumption). The results are shown in Figure 2.8; by halving the distance travelled by

Temperature (C)	Sheet Resistance (Ohm)	Standard Error	Temperature coefficient
25	20.77	0.035	-0.0097
30	20.90	0.061	-0.0143
35	20.77	0.026	-0.0028
40	20.57	0.013	-0.0048

TABLE 2.2: Recorded Electric Property of ITO-film

the charge carries, the time required for the heating element to reach 37°C is reduced by 49 %. The width of ITO-film remains unchanged at 29mm as it shares the same width value of PDMS microbio reactor, thus ensuring the PDMS micro-bioreactor is fully covered by the heating element.

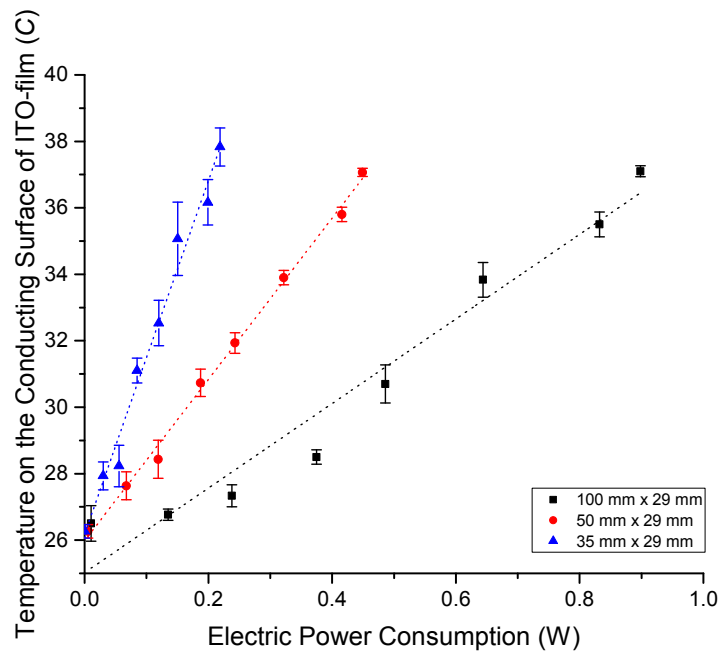


FIGURE 2.8: The Correlation between measured power consumption of different sized ITO-film and corresponding temperature (°C) on the film's surface

The ITO film with dimension of 35 mm × 29 mm and 50 mm × 29 mm ITO-films both reflect good linearity (R^2 equals 0.983 and 0.996 respectively) between the conductive

layer surface temperature and correlated power consumptions. However, the large magnitude of standard deviation error of $35\text{ mm} \times 29\text{ mm}$ ITO-film indicates a high level of inconsistency in reproducibility for proposed size of ITO-film as a heating element. The 29 mm width of ITO-film was kept the same during the experiments and changes were only applied for the length of ITO-film where two electrodes were fabricated at both ends. Therefore, changing the length results in a change in the distance and pathway of free charge carriers on the surface of ITO-film. Thus, the power consumption for the required temperature is linearly correlated to the travel distance of free charge carriers. Among the selected areas (travel distance), the $50\text{ mm} \times 29\text{ mm}$ ITO-film performed with highest linearity between surface temperature and corresponding power consumption, with least variability. Thus, this ITO-film will be used for future experiments.

2.3.1.2 Optical Property of ITO-film

The analysis was carried out under a visible light microscopic observation with $10\times$ lenses setup. Figure 2.9 shows the microscopic image for SH-SY5Y cell culture on day 3 in both Green and Red spectrum with and without conducting ITO-film. Figures 2.9 (a) and (b) indicate that under wavelength from 495 nm to 570 nm , the transmittance of the ITO-film is significantly reduced. In contrast, microscopic observation was unaffected in the wavelength region between 620 nm and 750 nm .

Further quantitative analysis was carried out to determine the image quality includes traditional method PSNR in dB, were obtained from the previously stated equation in Section 2.2.10 (Eqn. 2.13 and Eqn. 2.14) and algorithm of Structural Similarity Index (SSIM) can be found in Appendix A. Although the PSNR does not fully represent the transmittance (radiant energy) of ITO-film at specific spectrum, nevertheless it gives an indirect indication for the estimated level of material's transmittance by directly comparing each pixel's signal strength between two images recorded under same spectrum range. In imaging processing, an image with SNR value equal or higher than 32.04

dB is of excellent quality, whereas an image with SNR between 20 dB and 32.04 dB is acceptable. The PSNR(dB) were calculated and are illustrated in Table 2.1. With conducting ITO-film, the obtained microscopic image in Green spectrum is regarded as low quality image with PSNR value lower than 20 dB , whereas Red spectrum image is within the acceptable range.

Analysis such as PSNR and MSE estimate absolute errors; in contrast, SSIM assesses the visual impact of image quality based on an initial uncompressed or distortion-free image as reference. It is a perception-based model that considers image degradation as perceived change in structural information, while also incorporating important perceptual phenomena, including both luminance and contrast terms. The resultant SSIM index is a decimal value between -1 and 1, and value 1 is only reachable in the case of two identical sets of data. In our analysis, the microscopic observation with and without conducting ITO-film gained a SSIM of 0.88 under Red fluorescence spectrum while the SSIM reduced significantly to 0.42 under Green fluorescence spectrum.

	Red Fluoresce	Green Fluoresce
Spectrum wavelength	625 nm -750 nm	495 nm -570 nm
Structural Similarity Index (SSIM)	0.8757	0.4187
PSNR (in dB) with conducting ITO film	27.3516	18.17

TABLE 2.3: Analysed SSIM and PSNR for recorded microscopic image under Red and Green fluorescence spectrum

Furthermore, by comparing the images between b) and d), it can be observed that the transmittance percentage increases as the wavelength region shifted from Green spectrum to Red spectrum. Past studies revealed that ITO film, as a heavily-doped n-type semiconductor, has a transport/electrical energy bandgap that is temperature dependent [Unlu 1992]. By applying voltage difference on the side of ITO film, it increases the number of free moving charges on the film. As a result, the current is due to electron drift where those electrons collide with their atoms vibrating about their mean position

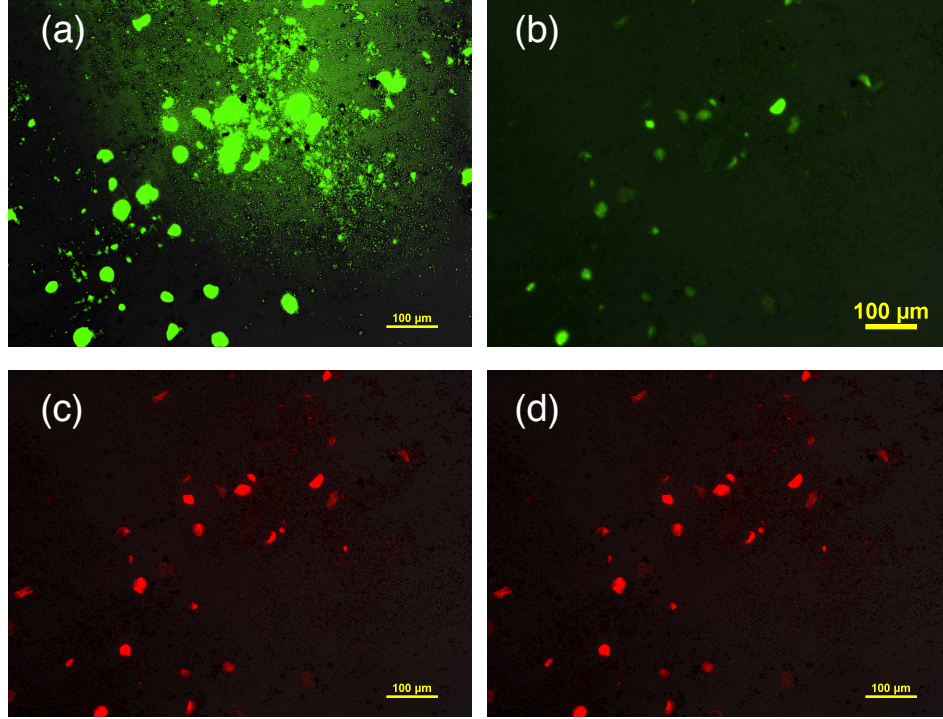


FIGURE 2.9: Microscopic observation for cell culture in PDMS with ITO films that firmly attached to the bottom of the microbioreactor. (a) and (c): Non-conducting ITO films without potential applied; (b) and (d): Conducting ITO films with applied potential.

and ultimately increases the amplitude of their thermal energy. This increase in thermal energy in turn causes further increases in amplitude of the atomic vibrations and hence interatomic spacing also increases. An increased interatomic spacing decreases the potential seen by the electron in the material, which in turn reduces the size of the electrical energy bandgap. The temperature dependence of the transport energy bandgap has been experimentally determined in the past yielding the following expression for E_g as a function of the temperature T (Kelvin) [Suresh 2010].

$$E_g(T) = E_g(0) - \frac{\alpha T^2}{T + \beta} \quad (2.15)$$

E_g , α and β are material constant.

In an organic semiconductor, there could be a distinction between photon absorption energy bandgap (optical bandgap) and transport energy bandgap as optical bandgap is at

a lower energy than the transport gap. For most types of inorganic semiconductor, such as silicon and ITO, there is very little interaction between electrons and holes. Therefore the optical and transport energy bandgap are essentially identical and the distinction between them can be ignored. Therefore, the decreases in transport bandgap, due to its temperature dependence characteristic, in ITO film may also reduce its optical bandgap for photon absorption. As a result, the fluorescence with high energy (short wavelength region 495 nm - 570nm), that passes the non-conducting ITO film without much of absorption, may be absorbed when it passes through a conducting ITO film. The analysis suggests that, considering the conducting ITO-film transmittance characteristics, green fluorescence dyes should be avoided as a bioassay in the relevant cell culture.

2.3.2 Sensing Element Characterisation

The sensing characterization was experimented with temperature fluctuation from 24 °C to 44 °C and repeated within 34 °C to 37°C range as this is most commonly used temperature range for consecutive heating and cooling processes.

Thermistor	Steinhart-Hart model linearity (R ²)	Temperature coefficient (average)	Correlation to TH-10K (R ²)
TH	0.994	0.421	N/A
JT	0.895	0.306	0.962
SP	0.990	0.380	0.990
GP	0.995	0.408	0.987

TABLE 2.4: Calculated physical parameters for different Thermistors

By utilizing the Steinhart-Hart model, which was explained in Section 2.2.5 (Eqn. 2.11), the experimental data was analyzed and the correlation between resistance for JT, SP and GP and TH-10Kmp thermistors were obtained with linearity of 0.895, 0.990 and

0.994 respectively. The Temperature Coefficient of resistance fluctuation was calculated for each type and is collectively displayed in Table 2.4.

Both the SP and GP thermistors respond linearly accordingly with Steinhart-Hart model and were equally correlated to the accurate thermistor TH-10K. A small difference in temperature coefficient between TH-10K and GP indicates that they share a similar change in physical property of the thermal sensor itself at a given change in temperature range between 34 °C to 37 °C. Thus, the GP thermistor is the best feedback sensor for accurate temperature control within the 34 °C to 37 °C interval.

2.3.3 Simulation and Optimization for Thermal Distribution on ITO-film Surface

The static (point-point) measurements on the surface of ITO-film were reported with lack of uniform thermal distribution and a number Infra-red thermography experiments and investigations were implemented.

2.3.3.1 Infra-Red Thermography

The obtained data for the thermal distribution of two unmodified ITO-film surface with area of 100 mm × 29 mm were illustrated in Figure 2.10 a) and b). It clearly demonstrates that, despite placing the supplier point in the middle, one end of the ITO-film can easily get more overheated than the other side. The major reason for this non-uniform distribution is because once a charge carrier was excited by a voltage difference, it tends to move freely on the ITO-film and will always choose the shortest path to move from one electrode to the other. As such, a uniform thermal distribution would require the two copper wires to be exactly perfectly parallel, which is practically not possible, especially for mass production.

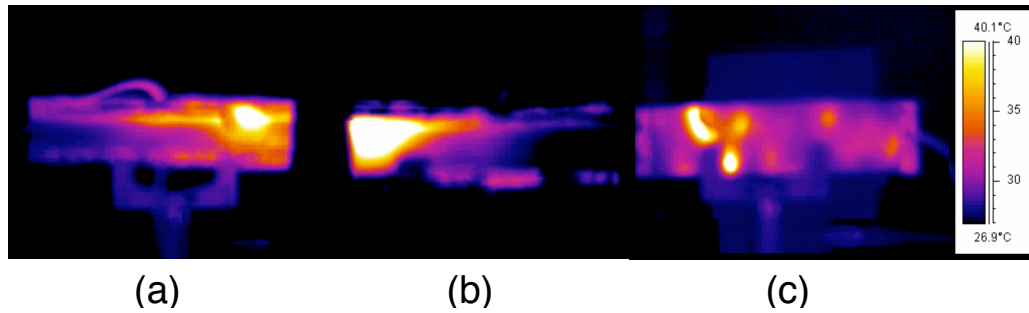


FIGURE 2.10: (a) and (b) Illustration of ITO-film with non-uniform thermal distribution; (c) Improved sample heating distribution with induced charge carrier pathway

To improve thermal distribute, a guided pathways for the free charge carriers to follow was imposed on the film's conductive surface. This helped to increase the surface coverage that is under heat flux flow and thus improves thermal distribution of ITO-film. An IR image of this zigzagging ITO film, or Z-ITO, with area of $100\text{ mm} \times 29\text{ mm}$ is illustrated in Figure 2.10 where the thermal energy is more evenly distributed with few overheated areas on the film. However, the charge carriers must travel 275 % more than before, thereby requiring more current to reach 37°C . As a result, the power demand is higher than a set of battery can provide, making it unsuitable for portable applications.

2.3.3.2 Numerical Simulation and Analysis

To examine the homogeneity of the thermal field on the developed ITO-film and in the chamber of PDMS microbio reactor, numerical simulations were carried out.

Four non-conductive elements with length in a ratio of 0.6 to its electrode length (i.e 14.4 mm) was added to the surface of ITO-film and were 9.85 mm distant to each other. Figure 2.11 (a), showed that an improved thermal distribution was partially achieved on the surface of ITO-film ($50\text{ mm} \times 29\text{ mm}$), but the heat flux movement is impeded by the last non-conductive element in the steady-state. Figure 2.11 (b) from Infra-red

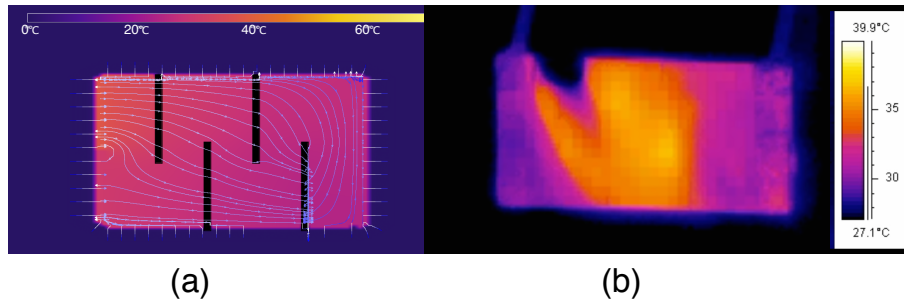


FIGURE 2.11: (a) Simulated results of improved ITO-film ($29\text{ mm} \times 50\text{ mm}$); (b) Infra-Red Thermography recording image of improved ITO-film ($29\text{ mm} \times 50\text{ mm}$) with illustration of an impeded thermal distribution on the film surface

thermography shows the impeded thermal distribution. The non-uniformity is believed to be caused by a small heat flux density along the carriers moving pathway.

An improved version of ITO-film was later proposed by minimizing the travel distance along the horizontal axis for charge carriers meanwhile increase the length of non-conductive elements and as a result it remains the overall travelled distance of charge carriers on the film surface unchanged at 93.78 mm where the optimal travel distance was obtained by utilizing equations described in Section 2.2.3.

The ITO film with dimension of $35\text{ mm} \times 29\text{ mm}$ was then chosen and operated under a constant 0.44 W power supply. The power source was repositioned to maximize the conducting coverage area on the surface of ITO. As illustrated in Figure 2.12 (a) and (b), which indicate that the position of the power source significantly impacts the thermal distribution. It is to be noted that, due to the impurity of ITO film, there is some discrepancy between the measured and simulated result. In the simulation, the Z-ITO heating element was assumed to deliver uniform watt density (heat flux) across the entire heating area. In practice, the electrical current travels the path of least resistance, the watt density in the ITO film decreases with distance from power connection points and there are losses particularly around the edges.

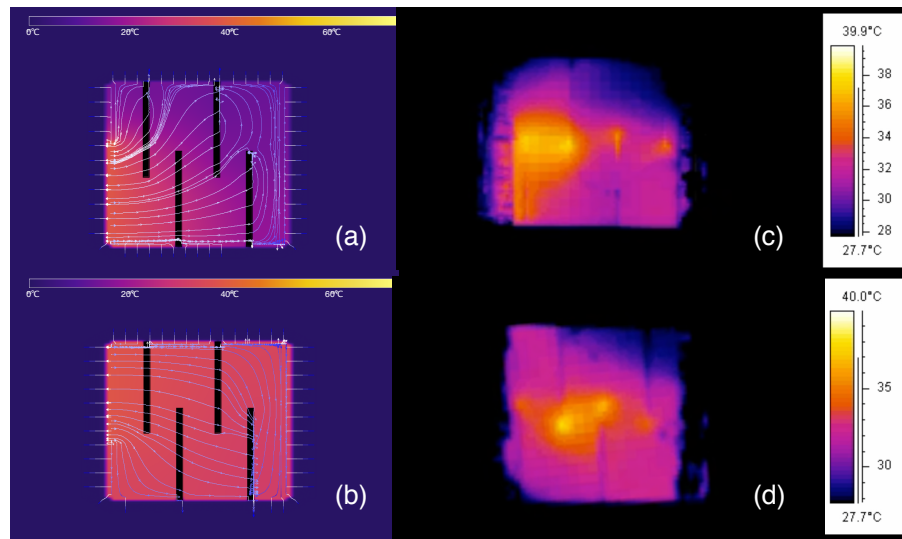


FIGURE 2.12: Power source position effect for thermal distribution on the surface of ITO-film with dimension of $29\text{ mm} \times 35\text{ mm}$ (a) A clear heat flux blockage; (b) No major blockage for charge carriers

A unique option with Z-ITO film is the ability to design the heater for extra power precisely where it is needed. By varying the distance within the pathway pattern, it is possible to put just enough extra heat on the edges to compensate for the losses. This is often referred as 'profiling' the watt density and is expected to obtain the optimum temperature across the entire surface without adding excess heat and it will certainly be an interesting avenue for future investigations. However, under this circumstance, by firmly attaching the Z-ITO film, as an heating element, to a material (PDMS) with low thermal conductivity and high thermal inertia characteristic, it may not be necessary to improve the thermal energy distribution of ITO film surface. Comparing Figures 2.12 (c) and (d), the Z-ITO film with repositioned power source did reflect a more evenly distributed thermal energy along the middle horizontal axis.

Experiments revealed the difference of thermal distribution was largely affected by the position of power source as demonstrated in Figure 2.13. In case (a) the majority of charge carriers are moving directly from the V+ position to V-, thus the far end of the busbar (copper electrode) has fewer charge carriers to move around, resulting in the

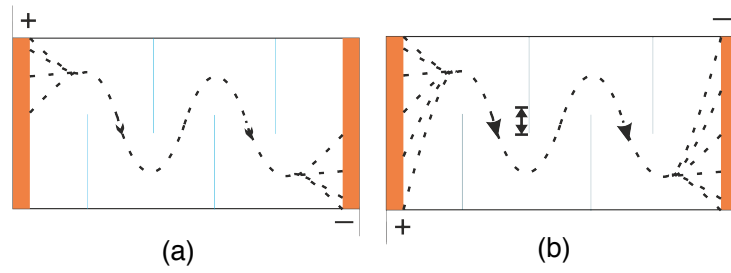


FIGURE 2.13: Illustration of power source positioning effect (a) Shorter pathway; (b) Longer pathway with improved thermal distribution

passing of no current. In contrast, in case (b), because the path for charge carriers is closer to $V+$ and is semi-blocked by the non-conductive element, the carriers are forced to move around; this movement causes the heat flux to cover large areas of the ITO film, resulting in an improved thermal distribution.

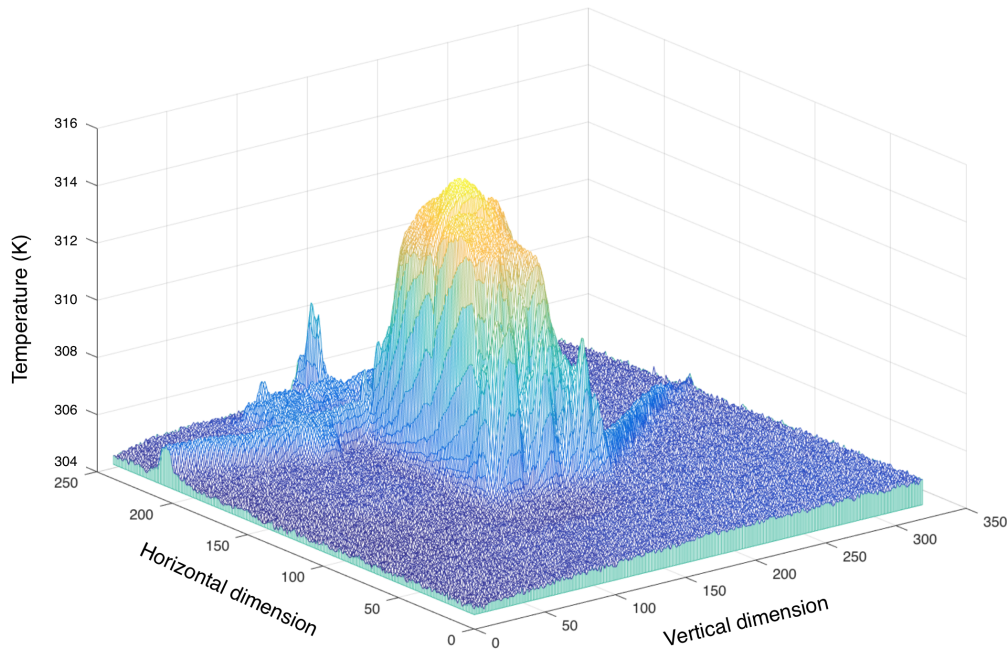


FIGURE 2.14: A 3D-mesh plot for thermal energy distribution on a single modified ITO-film ($29\text{ mm} \times 35\text{ mm}$) surface when heating element reaches its steady state which was controlled by switching relays

The distribution was further analyzed and the Figure 2.12 (d) was mathematically converted into a 240×320 matrix array prior to image processing. A 3D mesh plot was

obtained via the re-arrangement of matrix array and from Figure 2.14, the edge of ITO-film contains sharp temperature gradients, which indicate a high rate of temperature changes along the edge of ITO-film. The surface temperature fluctuated within ± 0.5 °C interval around 37 °C. Each value in the matrix represented the signal strength that is directly proportional to the thermal energy detected via IR thermography.

By having achieved an ITO film with improved thermal distribution and to carry out the further simulation with PDMS, it is necessary to mimic the cell culture conditions in the simulation. Therefore, the culture chamber was assumed to be filled by water with low fluid flow rate in the chambers was low and the heat transfer was assumed to be dominated by heat conduction. Furthermore the heat radiation was neglected and heat conduction within the liquid samples, PDMS chambers wall and ITO film was considered. A constant heat generation rate was assumed to apply on the ITO film.

The heat transfer phenomenon occurred in the proposed experimental setup (ITO film attached to PDMS) is mainly governed by the mechanism of thermal conduction which mainly occurs between ITO-film based heating element and PDMS microbioreactor as well as convective heat transfer which took place inside of culture chamber. There is a practical difficulty in determining the free convection effect as the convection heat transfer coefficient (h) is a function of many parameters including surface geometry, fluid motion and properties of fluid velocity etc. The typical values lies between $2 \sim 25 \text{ Wm}^{-2}\text{K}^{-1}$. In this case, the value of free convection is estimably evaluated as $7.5 \text{ Wm}^{-2}\text{K}^{-1}$ [El-Ali et al. 2004]. The boundary conditions and the thickness of each layer were also illustrated in Figure 2.15. The free convection in y-z and x-z plane and adiabatic condition in x-y plane were imposed on the surface of microbioreactor chip. Under adiabatic condition (x-y plane), the energy is transferred only as work and overall heat transfer across the boundary between thermodynamic and surroundings is absent. The non-uniform mesh grids were applied for these numerical simulations. A residual criterion was used to guarantee the convergence of the solution where its value was set

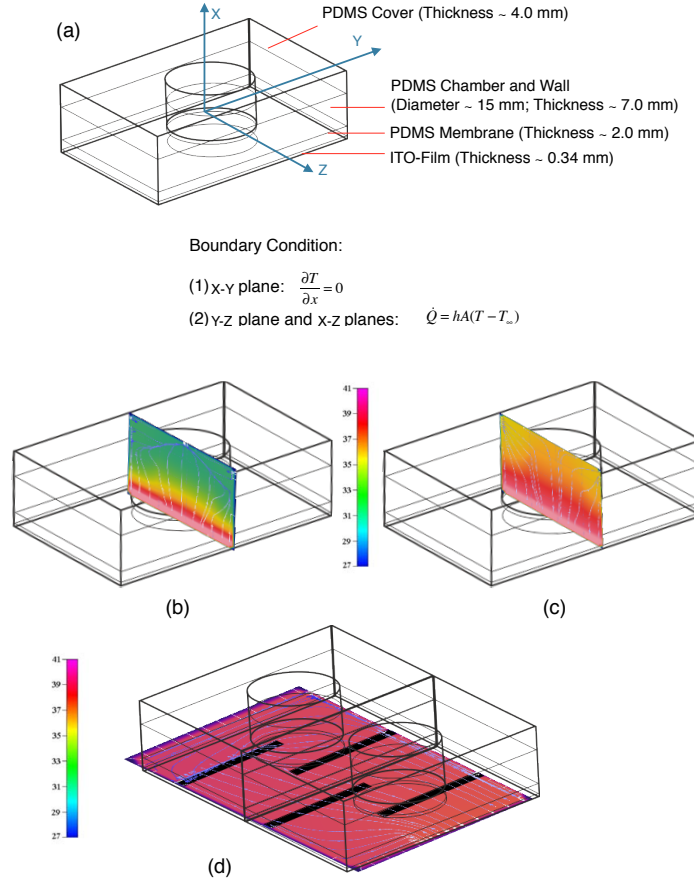


FIGURE 2.15: Numerical simulation-based evaluation of (a) each layer of materials and boundary conditions, of the temperature ($^{\circ}\text{C}$) distributions; (b) in the PDMS microbioreactor chamber at initial condition; (c) in the PDMS microbioreactor chamber at steady state which is controlled by switching relay; (d) on the ITO-film which is firmly attached to the bottom surface of PDMS microbioreactor

to be less than 10^{-6} . With respect to the simulated thermal field in the chamber of microchamber, Figure 2.15 (c) revealed the thermal distribution in the x-z plane in the microbioreactor chamber at steady state. It can be clearly seen from the result that a spatially homogeneous thermal condition can be realized in the chamber for cell culture. Figure 2.15 (d) showed that a uniform temperature distribution was achieved on the surface of ITO microheater chip in the case of $q = 1.5 \times 10^5 \text{ Wm}^{-3}$.

Further experiments also show that the low thermal conductivity of PDMS favoured

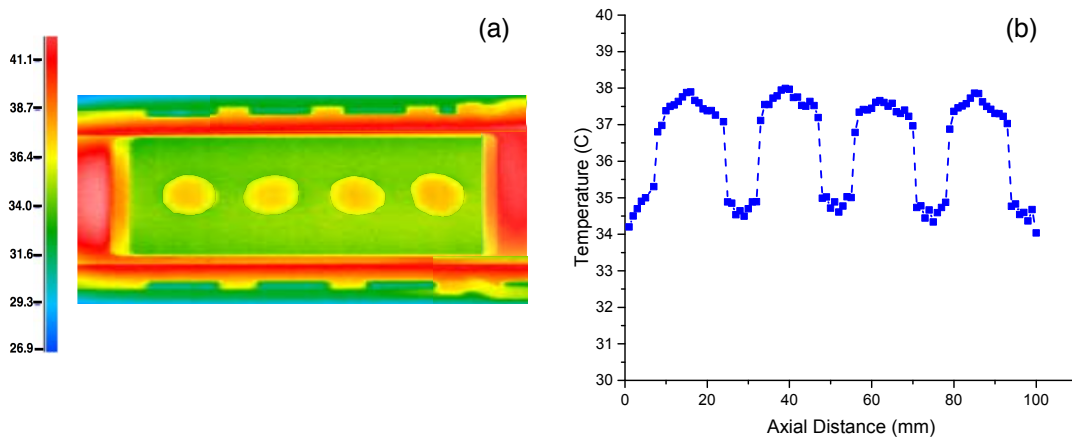


FIGURE 2.16: (a) 2-dimensional top view IR images (Matlab) of PDMS microbio-reactor with two ITO-films attached to its bottom side ; (b) The comparison of the temperature profile of the four microbio-reactor chambers (The IR images has 320×240 pixels and the resolution of each pixel is 500 μ m)

the improvements of thermal distribution that was generated by the ITO-film heating element. It also added a damping factor in the feedback control algorithm to reduce the fluctuation and oscillation of the proposed system. This outcome is, basically, in line with the previous thermal simulation (refer to Figure 2.15). Besides, to compare the temperature field occurred at the 4 microbio-reactor chambers in the PDMS microbio-reactor platform, Figure 2.16 profiled the temperature distribution along with the central axial distance. This result revealed that the temperature field in the 4 microbio-reactor chambers was identical with a slight variation of ± 0.8 °C and with ± 0.3 °C temperature variation for each chamber filled by culture medium, which is acceptable for cell culture application.

2.3.4 Thermal System Validation

To examine if the thermal condition is homogenous in the microbio-reactor culture chamber and to determine the parameters in the feedback control algorithm, further IR imaging was carried out where the PMDS microbio-reactor is attached to the proposed ITO-film with desired size and power. Each chamber was filled with culture medium

to mimic the real cell culture condition. The proposed heating system is illustrated in Figure 2.7. The results show that even when the temperature of ITO film began to drop, the temperature in the culture chamber with air was still heating up inside of PDMS. The propagation delay of temperature turning point between ITO film and the turning point in culture chamber was non-linear in the first few heating cycles. This non-linearity tends to get smaller as the order of the switching cycle increases. Due to this non-linearity of the propagation delay, the indirect temperature monitoring is not feasible to be implemented by measurement of film's surface temperature. In contrast, the embedded thermistor with 1.5 mm distance to culture chamber suggested a one-to-one relationship between current and temperature. A further analysis was carried out with culture medium, and it was found that the ratio between measured temperature in the culture chamber and estimated temperature from the embedded GP thermistor via Steinhart-hart model can be utilized to collectively establish a simple proportional-parameter based correlation which is shown as following:

$$T(R)_{nth} = \begin{pmatrix} a \times t + b \times \frac{1}{A+B\ln(R)+C(\ln(R))^3}, n = 1 \\ c \times \frac{1}{A+B\ln(R)+C(\ln(R))^3}, n \neq 1 \end{pmatrix} \quad (2.16)$$

$$Ratio_{nth}^{heat} = (-3.84 \times 10^{-3}) \times n + 1.145; \quad (2.17)$$

$$Ratio_{nth}^{cool} = (-6.72 \times 10^{-3}) \times n + 1.161; \quad (2.18)$$

$$T(R)_{nth}^{heat} = Ratio_{nth}^{heat} \times T(R)_{nth}; \quad T(R)_{nth}^{cool} = Ratio_{nth}^{cool} \times T(R)_{nth} \quad (2.19)$$

Moreover, from the multi-cyclic thermal experiment analysis, it was found 36.8 °C to be the critical value in heating process, as the temperature in the well will continuously increase up to 37 °C and maintain this temperature for at least another 85s due to the low thermal conductivity of PDMS after switching-off the power supply to the system. The evaluation also suggest that once the temperature in a culture chamber reaches 36.7°C, the power should be switched on in order to keep the temperature of the well in a

range of 36.6-37 °C. These two values obtained from large number of experiments have been used in the simplified PID control system.

2.3.5 Automated System Integration and Validation

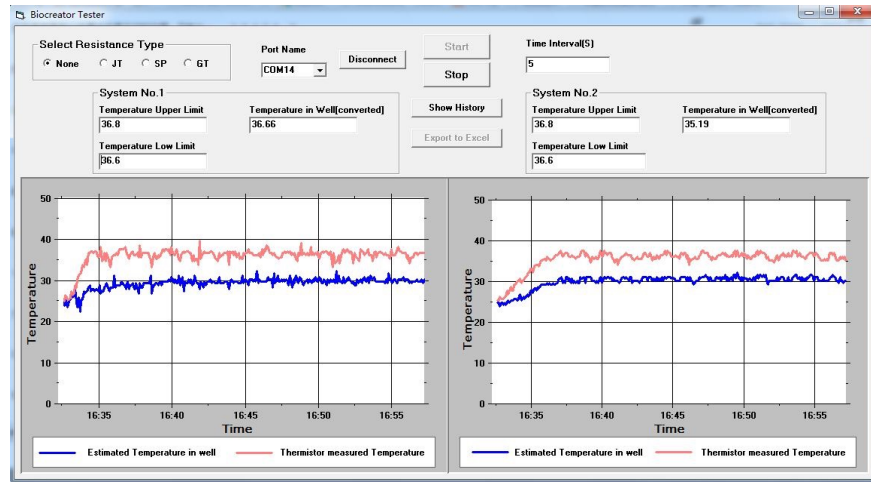


FIGURE 2.17: Developed GUI Interface for controlling ITO-film based heating system

The earlier sections showed ITO film as an efficient and reliable heating element, which can offer an improved thermal distribution across its surface in the microbioreactor chamber with a temperature of 37 °C with little fluctuation of ± 0.3 °C. Combined together with a characterized thermal sensing element, switching relay unit, a microprocessor unit, as well as the control principle, an automated system was realised. Figure 2.17 demonstrated the developed GUI for the proposed heating system, where red line is the converted temperature value from the embedded thermistor and the blue line is the estimated temperature in the culture chamber. It can accommodate different types of characterized thermistors, including TH, JT and SP.

The long duration temperature monitoring via the proposed automated system in PDMS chamber with air and culture medium were separately implemented and repeated three

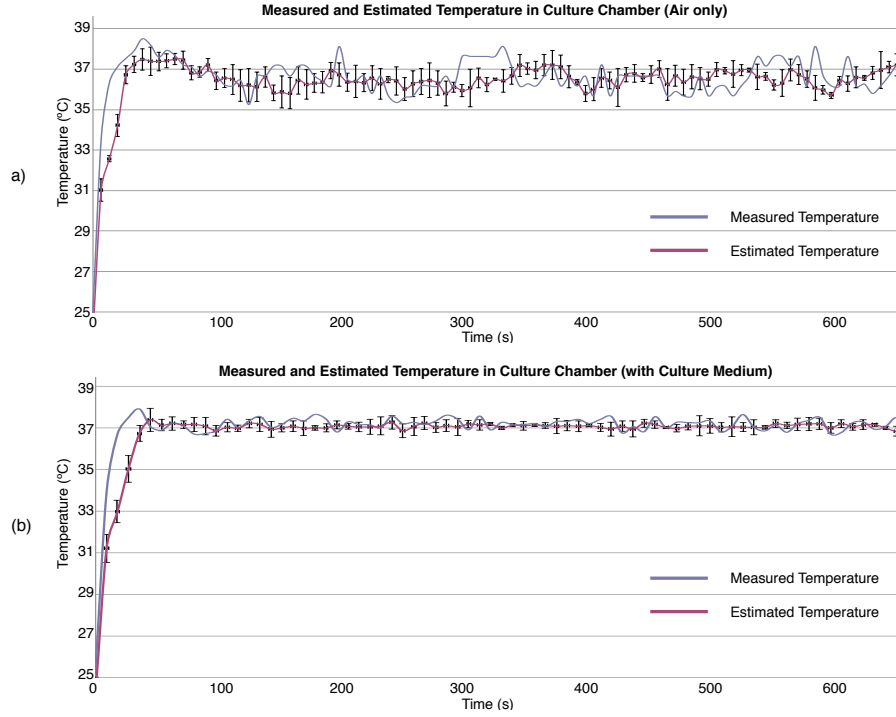


FIGURE 2.18: Measured and Estimated temperature in microbioreactor culture chamber (a) air; (b) culture medium where the chamber with medium illustrates a significant damping effect

times (shown in Figure 2.18 (a) and (b)). Both figures show the recorded temperature and estimated temperature via the embedded GP thermistor over 10 hours. A histogram of the culture chamber temperature is illustrated in Figure 2.19 for air and medium separately. The culture chamber with medium (which has larger thermal conductivity) reduced the temperature fluctuation in comparison to air. The fluctuations in temperature are strictly within ± 0.3 °C in all experiments. The power consumption was recorded to be $0.44W$ over 10 *hours* measurements, thus a 9V lithium battery with 1200 mAh can continuously supply the system for up to 21.34 *hours*.

This confirms that the low thermal conductivity of PDMS filled with medium allows further improvements of thermal distribution that was generated by ITO-film heating element. It also added a damping factor in feedback controlled algorithm to reduce the fluctuation and oscillation of proposed system. This system enables one to determine the temperature in a culture chamber indirectly and non-invasively.

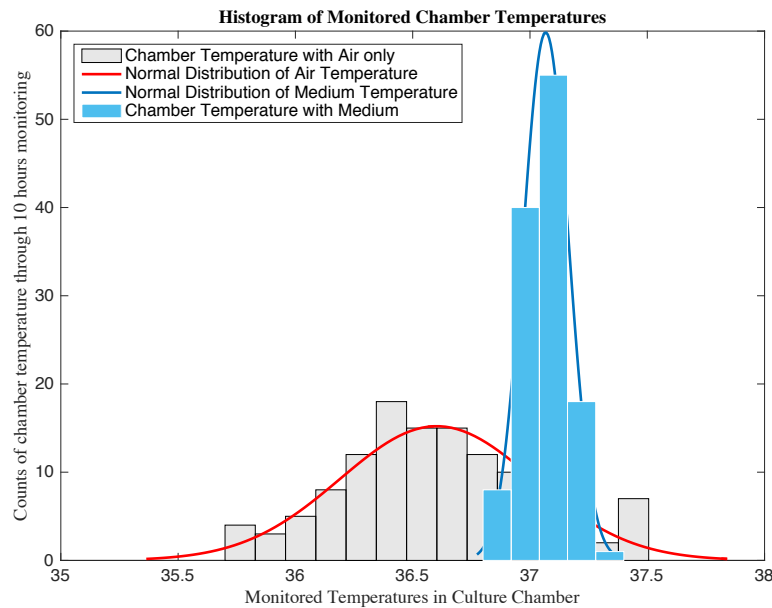


FIGURE 2.19: Histogram of measured temperature in culture chamber with air (Grey) and culture medium (Blue)

2.3.6 SH-SY5Y Cell Culture

The capabilities of the proposed ITO-film based heating system to provide precise temperature control and improvements of thermal distribution have been discussed and demonstrated previously. The attempt of the proposed ITO-film portable device is to take the role of conventional bulky incubator for cell culture in order to improve the integrability with potential experimental setup including on-site microscopic observations and real-time cell number monitoring which will be explained in Chapter 3.

SH-SY5Y cells are a useful human cell line for reporting on the establishment of culture conditions, therefore a four day static cell culture of SH-SY5Y cell using PDMS micro-bioreactor platform, TissueFlex[®] [Cui 2007] was implemented to validate and verify the thermal environment offered by the automated system compared with conventional incubation. Viable cells glowed red when excited with 568nm light, due to the expression of mCherry protein.

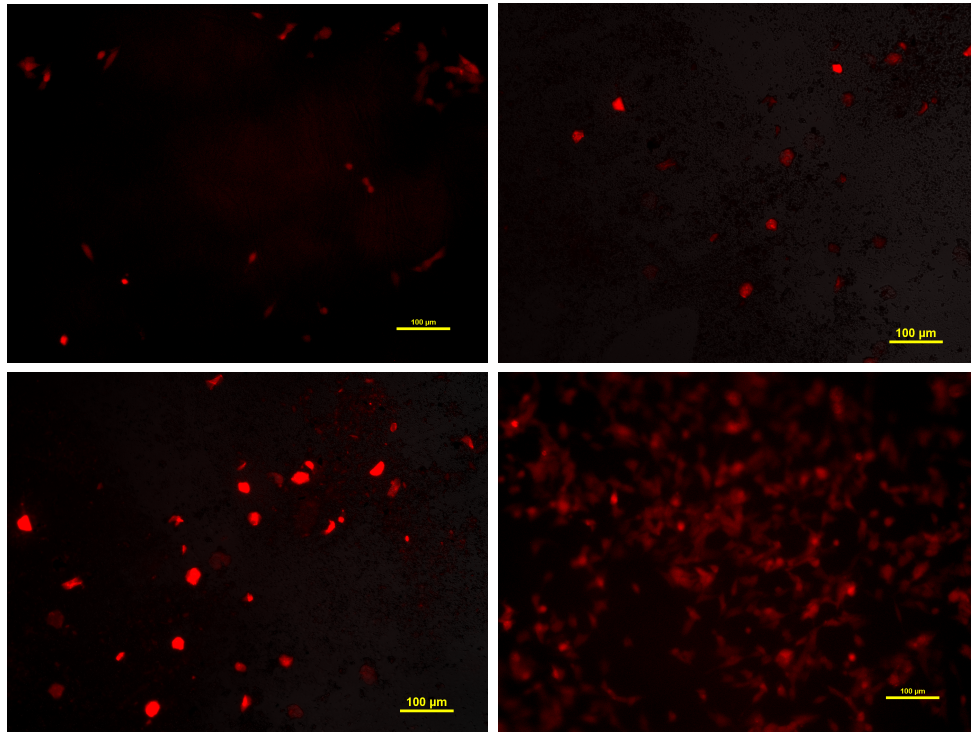


FIGURE 2.20: Microscopic observation of SH-SY5Y recorded over 4 days time-lapse cell culture experiments (a) 1st day; (b) 2nd day; (c) 3rd day; (d) 4th day

The Figure 2.20 illustrates the microscopic observation for consecutive 4 days static cell culture for ITO-film based system. The total cell number under different thermal conditions include ITO-film based heating system, conventional incubation and under normal room temperature, were all counted via microscopic observation and each total cell number value was obtained by averaging the counting number on 5 separate microscopic images in Red spectrum channel only as the conducting ITO-films reduced the transmittance percentage significantly in the green channel. The statistical results, which is shown in Figure 2.21, exhibited that the exposure of SH-SY5Y cell to the room temperature in PDMS microbio reactor causes the cell decreases its total cell number dramatically after two days cell culture. Despite the gas permeable area was reduced by adding an ITO-film at the bottom side of PDMS microbio reactor, the proliferation rate was not significantly affected. Hence it can be concluded that an external oxygenate culture medium through microfluidic channels with a precisely controlled laminar flow rate could be helpful to facilitate the cell culture, but may be unnecessary.

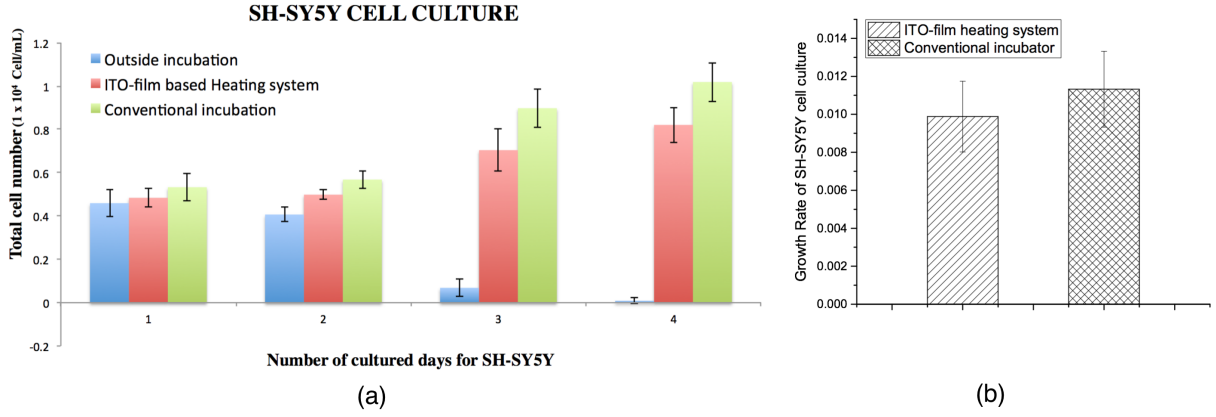


FIGURE 2.21: (a) Total cell number estimated via microscopic observation for SH-SY5Y cell culture in three thermal conditions compared during 4 days static cell culture. Results are given as mean $\pm$ standard deviation of three separated experiment ($n=9$); (b) Growth rate of 4 days static SH-SY5Y cell culture in ITO-film and conventional incubator. Growth rate is defined as amount of doubling in one unit of time

The tendency of static cell culture with ITO-film system is clearly stated from the data by increasing its overall estimated total cell number with 69 % in 4 days culture in PDMS. The results are given as mean $\pm$ standard deviation of three separated experiments ($n=9$). The calculated growth rate (amount of doubling in one unit of time) over 4 days static cell culture in two thermal controlled environments demonstrated no significant difference ($p = 0.450 > 0.05$), with level of $9.88 \pm 1.86 (\times 10^{-3}) h^{-1}$ and $11.32 \pm 2.00 (\times 10^{-3}) h^{-1}$ for SH-SY5Y cell culture on the ITO-film platform and in a normal incubator respectively. As a whole, within the experimental conditions explored, this evaluation demonstrated that the proliferation rate of the cultured SH-SY5Y on the developed ITO-film based system is consistent with that of in a conventional cell incubator.

2.4 Summary

In this study, a transparent ITO-film based heating system with a large heating area was proposed with the attempt to serve as a thermal control system for long term

static cell culture outside the conventional incubation area. A prototype of a labour-free portable, transparent, energy efficient monitoring and control system for cell culture in a microbioreactor that is outside of an incubator and is able to carry out parallel experiments under differently required temperature conditions has been constructed. It enabled on-site microscopic observation over the cell culture process through a selective spectrum. This was achieved through an indirect thermal determination method using a PDMS microbioreactor, which is highly gas permeable and transparent, and a novel ITO (Indium Tin Oxide) film as a transparent heating element.

An improved thermal distribution over large ITO-films was achieved through characterization and investigation of its properties through numerical simulations and experimentation. Different from the conventional wet etching screening technique, the proposed ITO-film heating element is cost-effective and simple. The reliability and characterization of thermal sensing elements were also discussed and a cheap and scalable thermistor, GP, was chosen to act as the feedback sensor in a simplified PID control system. The long term monitoring proved the system allows the temperature of the microbioreactor to be controlled within a tolerable error of $\pm 0.3^{\circ}\text{C}$, which is suitable in general for cell cultures. The SH-SY5Y cell culture experiment followed with viability assay analysis has further revealed that the proposed automated system was able to maintain a stable thermal microenvironment that was comparable to conventional incubation. The proposed ITO-film based heating system, with indirect and non-invasive monitoring, has distinctive advantages in optical transparency and portability, making it an innovative portable technology for cell culture.

Chapter 3

Miniaturised Bioimpedance Spectroscopy in-situ Monitoring System for Cell Viability in Microbioreactor

3.1 Introduction

Precise cell culture monitoring is a key requirement for success in tissue engineering, which is often summarized as the ability to quantitatively measure and identify the stage of the culturing process; these are the proliferation rate, viability and metabolic status of cellular substrate, as well as its physiological and pathological changes. Conventionally, by-products of the metabolic process were used to determine cellular growth rate. The majority of those cellular analysis were performed in central laboratories as they require skilled personnel and specialised equipment. As a result, optical analysis via microscopic observation is the most popular method to determine the culturing process for cell culture in microbioreactors.

3.1.1 Existing Cell Culture Monitoring Technique

Cell culture studies often provide valuable information on the cells physiological condition and by extension the tissue and organism from which the cells originate. In general

cell culture-based assays can be broadly classified as either label or label free, where label based techniques require elaborate sample preparation, pre and post treatment.

3.1.1.1 Optical Inspection for Cell Culture Monitoring

The most widely used method for evaluating and quantifying cell adhesion and spreading during culture process, is based on the microscopic observation with fluorescent reagent. In principle, the cells are seeded onto a surface and allow the cells to adhere and spread for a length of time. A prepared buffer solution is then used to wash away the unbound cells in cellular substrate. The fully attached cells are labelled with fluorescent reagent and observed via an inverted microscope. The cells can also be pre-labelled with a fluorescent dye. Alternative cellular substrate could also be counted under light (bright-field) microscope with freshly stained cells.

Optical methodology can be used to monitor the process non-invasively, and the cellular substrate state and concentration can be obtained directly from the culture chamber without the need for sample removal and direct contact. Other major advantages of the optical system is the simultaneous recording of a selected wavelength and signal intensity. The fluorescence, absorbance, refractive index, colourimetry, and bioluminescence are commonly known as optical methods for biological analysis. The normal fluorescence is the most sensitive and popular technique, since it can reliably measure the viability of cells as the cells was tagged with fluoreophores such as calcein and propidium, whereas Lai et al. (2008) and Hellmich et al. (2006) published a number of reviews of laser induced fluorescence detection for microbioreactor.

Despite the popularity of the above-mentioned optical methods, there are some limitations. Firstly, most of the biological culture medium, and the PDMS used to fabricate microbioreactors are both highly fluorescent in Red channel, thereby interfering with the fluorescence analysis. For instance, the transmittance rate for transparent heating

elements, Indium Tin Oxide film, were reported to be low below 400 nm wavelength range; thus it is difficult to observe the cell culture over a microscope in the Green spectrum range when ITO is conducting. PDMS, which was to be used for the micro-bioreactor, in turn interferes in the Red spectrum range. Thus when the ITO film and PDMS micro-bioreactor were combined together and observed under a microscope, both excitation and output fluorescence were attenuated. Therefore, signal amplification and system gains need to be carefully tuned and will result in reduced sensitivity.

One of the other critical limitations is that most optical analysis methods, with the exception of Raman Spectroscopy, are based on the microscopic observations where the detected results may not be representative of the entire culture chamber. The estimation of total cell number and viability was obtained by averaging 5 ~ 10 microscopic captured images, where the different location of culturing sample could be significantly different and hence it cannot offer a quantitative identification of biological status.

3.1.1.2 Bioassay for Cell Culture Monitoring

The inaccuracy and the unrepresentative nature of the obtained data indicate the limitation of optical inspection for cell culture monitoring and this lack of quantitative character may be overcome by utilizing electrochemical methods.

On the other hand, the staining assays such as ethidium homodimer-1 (ETHD-1), Trypan Blue and Alamar Blue, have been reported to have several limitations. All staining were based on the principle of selected compound permeability to a specific cells membrane. The ETHD-1 may enter those dead cells and bind to DNA but it cannot enter the live cells since it does not have a compromised membrane [Zhao 2013; Sato et al. 2001]. Furthermore, the ETHD-1 relied on microscopic observation where results can be inaccurate. In contrast, the Trypan Blue staining is a vital stain, as illustrated in Figure 3.1, used to selectively colour dead cells blue while the live cells with intact cell membranes

are not coloured [Strober 2001]. The subjective evaluation with hemocytometer reduces the reliability of Trypan Blue viability assay. Additionally Trypan Blue assay is also heavily dependent on the precise human operations since the cells can be easily lost in transferring suspended solution from one container to another (i.e centrifuge tube to petri-dish and PDMS and vice versa). Uncertainties in cell concentration could also be introduced where the volume added to the cells when using pipette may cause a more dilute solution of cells and thus swing the results of cell viability assay. All of these

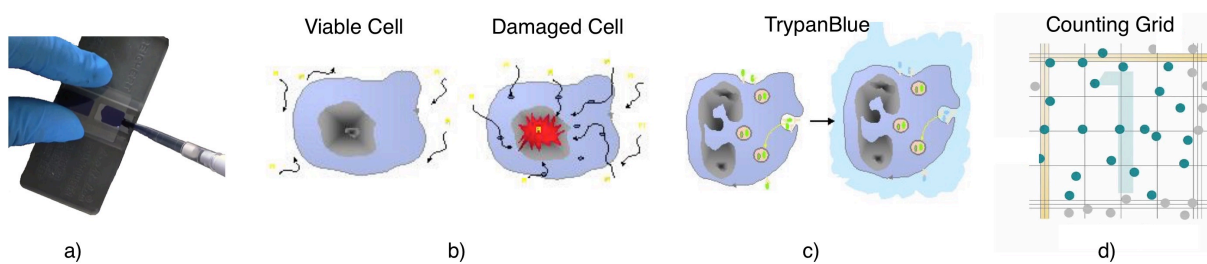


FIGURE 3.1: Illustration of a existing Trypan Blue bioassay. a) Illustration of adding Trypan Blue solution into cell sample where the samples were pre-loaded into the hemocytometer; b) Trypan Blue solution would only penetrates through cell membrane if and only if cells is damaged or dead; c) Demonstration of dye binds to intracellular proteins of leaky cells; d) Illustration of cell counting on a single square grid from dual-chamber hemocytometer where each chamber is divided into 9 squares. Image modified from On-Line, refers to Website-List

aforementioned techniques are described as endpoint assays as they only provide one-time information about the cell system [Grossberg et al. 2001] and are destructive to physiochemical microenvironment, bringing the biological experiments to an end [Li et al. 2003]. Moreover, pre-labelling, post-labelling, fixation and permeabilisation are all required and the peptides, dyes or proteins can potentially bring interference for signal transduction as they have been reported to introduce background noise to the measured data. On the other hand, although the evaluation for cell culture via microscopic observation allows rough estimation and continuity for further biological culture without staining or fluorescent treatment, the accuracy is fully dependent on the representativeness of the microscopically recorded image and cannot thus be guaranteed.

Thus there is a clear demand for non-destructive technique that can provide real-time information on cellular activity is highly desirable as it can provide a wealth of information about the biophysics and biochemistry of cells. The advances of microtechnologies and microelectrode-based devices in recent the decade improved the spatial resolution in the study of cellular processes. Among the potential microtechnologies, the Bioimpedance Spectroscopy technique has been intensively applied in impedance-based [Dodde et al. 2015; Aberg et al. 2010; Park et al. 2009; Thielecke et al. 2004] and electrochemical characterisation of cell culture [Doldán et al. 2016; Capaldo et al. 2016; K'Owino and Sadik 2005; Johnson 2003; Frew 1987].

3.1.1.3 Electrochemical Methods

Unlike optical methods where the signal output is dependent on detection area and volume, the electrochemical detection system are dependent on the electrode material and the surface area [Canali et al. 2016; Metshein and Rauno 2016]. In terms of system integration, the electrochemical micro sensors were integrated by soft lithographic methods where the grooves and channels are created during the fabrication process to accommodate the electrodes in position, followed by sealing the electrodes with another polymer layer. This approach is summarized in a number of reviewed publications [Wei et al. 2009; Hilml and Luong 2000]. The major drawbacks of electrochemical analysis are (i) they are normally operated in central laboratories ii) incapable of providing information on specific cellular activities that are directly linked to specific cell functions, biomarkers or signal pathway.

Critically, the electrodes in electrochemical analyses were only seen as tools, without considering its potential to harm or interact with the biological substrate. In fact, the interaction of sensor with the surrounding biological medium has been a critical challenge particularly in the application of time-lapse in-situ monitoring. It not only introduces a

non-linear offset in consecutive scans hence drastically reducing a measurements accuracy, but also absorb protein and cells onto the electrode surface.

To date, there have not been much of study to my knowledge of a time-lapse cell culture where electrodes determine proliferation directly in microbioreactor without a significant loss in sensor functionality. Thus there is an urgent need to examine the consistency and sustainability of the electrodes performance in desired biological content, where Cyclic Voltammetry analysis is essentially required.

3.1.2 Cyclic Voltammetry Inspection Method

It is previously reported that the processes in a bulk of biochemical solutions (ionics) are very different from the processes at the surface of electrodes (electrodics) [Bard and Mirkin 2012; Li 2011]. It is clear that any interface will disrupt the electrolyte solution and for electrodes, which are under potentiostatic control, there will also be an additional influence of the charge held at the electrode [Adams 1969]. These phenomena give rise to a region named the electrical double layer. During the past, among many developed models and theories, the Helmholtz, Gouy-Chapman and Stern were most commonly utilized to analyze the above-mentioned interfacial phenomenon [Bard and Faulkner 2000].

3.1.2.1 Helmholtz Model and Gouy-Stern Theory

At the electrode-solution interface, the transformation from electric to ionic conduction occurs. The electrode metal act as a source or sink of electrons and when the metal is wetted in the solution, an electric double layer is formed at the electrode surface [Adams 1969]. From past observations, the potential drop in electric double layer region appeared to be in a linear manner between two plates, where one plate is represented by the charges on the metal and the other plate by the ions at a minimum distance

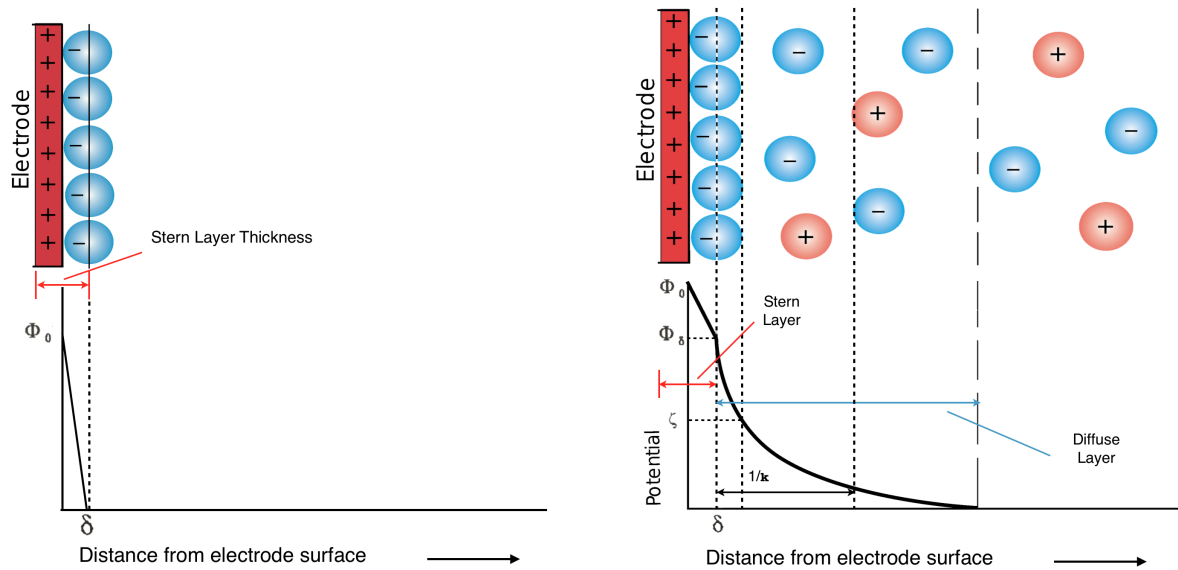


FIGURE 3.2: Helmholtz (left) and Sterns (right) model of electric double layer and electric potential as a function of distance from the electrode surface.

in the solution. The electric double layer is mathematically modelled as a function of the distance perpendicular to the surface of an electrode, which is shown in Figure 3.2 (a), and displayed the impedance response in a capacitive manner. Due the fact that the capacitive impedance value is inversely proportional to the distance between the 'plates' and the distance between the 'plates' were reported in the order of only ca. 0.5 nm [Monk 2001], the capacitive impedance value can be foreseen to be huge and exist intrinsically on a wetted metal electrode.

However, the Helmholtz model does not account for many factors such as, diffusion/mixing in solution, the possibility of absorption on to the surface, the interaction between solvent and the electrode [Murray 1996]. As a result, Helmholtz model cannot be applied to an electrode interface in a cellular culture medium condition. A combinational model of Gouy-Chapman and Stern theories was later introduced to address some of these limitations. The potential drop that occurs over the region is re-defined as the diffuse layer, where the layer is divided into an inner layer (Stern Layer) and an outer layer (the Gouy Layer), which is illustrated in 3.2 (b). It shows the Stern Layer is separated from the Gouy Layer at the Stern plane at a distance from the surface and this distance

is estimably correlated to the radius of ion [Monk 2001]. The surface potential of an electrode is high so that a significant part of the potential drop occurs in the Stern Layer, which is helpful in identifying the reversibility of an newly created electrode interface between an electrode surface and cultured cellular substrate under an oxygen permeable microenvironment. Further discussion of the Stern theory is outside the scope of this thesis.

3.1.2.2 Three-Electrode Monopolar Data Acquisition System

The three-electrode monopolar system is commonly used for the electrode-solution interface analysis. Monopolar system implies that the effect is determined by only one electrode whereas the other two electrode becomes the indifferent or neutral electrode [Murray 1996]. Previous literature has reported that the resultant current of a two-electrodes measuring system is dependent on the impedance of both electrodes plus the bio-impedance of cellular substrate. Furthermore, there are difficulties in estimating a possible contribution from the neutral electrode and difficulties to work with a sufficiently large neutral electrode in some cases. A third neutral electrode is therefore added to strictly control the measured cellular substrate to precisely determine the impedance of both electrodes. The setup is equivalent to the two-port three-terminal system with one shared electrode for both current injecting and voltage recording. The system can be simplified effectively with a practical setup contains an operational amplifier, which is illustrated in Figure 3.3, where the working electrode, W , and counter electrode, C , are the current carrying electrodes and reference electrode, R , which has no current flow and no polarization, effectively determines the potential difference with respect to working electrode, W . Considering the design principle of an operational amplifier, this circuit guarantees the potential at the reference electrode is equal to excitation voltage with constant amplitude [$v^+ = v^-$]. Thus the changes of measured potential difference

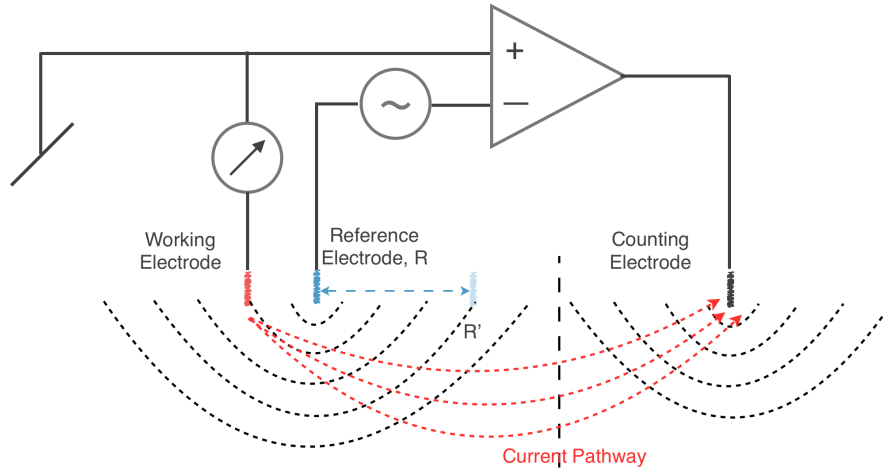


FIGURE 3.3: A simplified Three-Electrode Monopolar data acquisition system

with respect to working electrode is largely dependent on the changes of working electrode impedance which can only be caused by the creation of interface on the surface of electrode.

To determine the change of impedance under monopolar data acquisition system, the admittance, which is the inverse of impedance, needs to be measured. Generally, the admittance Y contains both the polarization of impedance of working electrode, Z_{ep} , and a cellular substrate volume zone proximal to the working electrode, Z_{CUS} , which is delimited by the position of reference electrode. The measured proximal or constructional cellular substrate zone corresponds to the equipotential surface shown as a stippled line under the reference electrode. The closer the distance between reference electrode and working electrode, the smaller the proximal zone of the current path. The transfer function of monopolar data acquisition system can be expressed as following and the derivative can be found in Appendix C.

$$\left(1 - \frac{Z_{ep}}{Z_{FB}}\right) = \frac{V_{out}}{V_e} = \frac{V_{out}}{V_{In}} \quad (3.1)$$

The bioimpedance of the cellular substrate, Z_{CUS} , has been mathematically eliminated.

Mirtaheri et al. (2005) suggested the polarization of counter electrode and the impedance of reference electrode will not influence the results. Nevertheless, the position and local sensitivity of electrodes does affect the volume of cellular substrate in the measured zone and it may change the local current density.

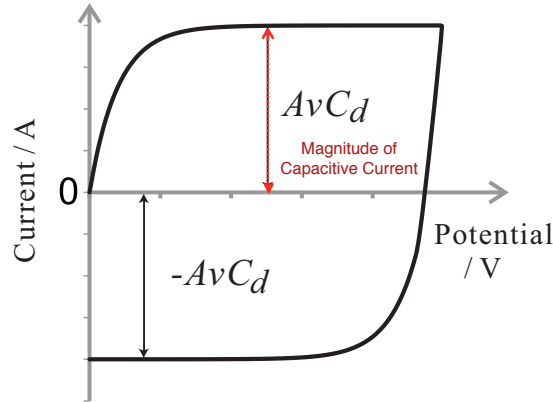


FIGURE 3.4: Current-potential plot resulting from a cyclic voltammetry analysis to a RC circuit

$$I = vAC_d[1 - e^{-\frac{t}{AR_sC_d}}] \quad (3.2)$$

The Cyclic Voltammetry response analysis was carried out in this thesis by utilizing this three electrode monopolar data acquisition system. This system was used to investigate biocompatibility of selected electrodes, such as reversibility of newly formed interface between electrodes and cellular substrate, the magnitude of capacitive current (illustrated in Figure 3.4), Faradic current, to precisely identify the conditions for triggering negative effects by polarized electrodes. The capacitive current expression is shown as Eqn. 3.2 and its derivation refers to Appendix B. Ultimately, this will eliminate the potential negative effect or at least reduce the possible effect to a controllable level during long term cell culture.

It is to be noted, previous literatures have suggested the cell culture environment tends to be more stable and homogenous during the entire culture period by introducing the perfusion system which continuously supplies nutrients and removes waste in the well

[Lei et al. 2014; Wu et al. 2007]. From cell culture perspective, the perfusion based culture system not only favours cell expansion and differentiation, but also provides a laminar flows of metabolised medium that exhibits some physiological information for potential further analysis[Zhang et al. 2006]. In contrast, the conventional static cell culture is either in a closed or semi-closed system where oxygen and carbon dioxide are exchangeable, but the medium is replaced on a periodic basis which not only brings the risk of contamination due to manual intervention [Naderi 2011], but it also favours the formation of a fluctuated and unsteady culture environment [Titmarsh et al. 2011; Wen et al. 2010; Wu et al. 2008; Wu et al. 2006]. Thus the perfusion-based microbioreactor system has thus gained popularity in the field .

However, the perfusion-based system supplies dissolved oxygen continuously to the culture chamber, which will introduce reactive oxygen species such as hydrogen peroxide into the culturing system. The reactive oxygen species were reported to facilitate interaction between selected electrodes and cellular substrate [Mercanzini et al. 2009; Keefer et al. 2008; Rahman 2007; Lingane 1996]. These interactions can accumulate over time and hinder the precise quantification of on-site electrochemical monitoring and analysis. Thus despite its popularity, the in-situ monitoring investigation via perfusion-based cell culture system is out of scope in this thesis.

3.1.3 Bioimpedance Spectroscopy Inspection Method

The optical and bioassay-based monitoring system are both time-consuming and require intensive labour operation during culture process and are difficult to implement in high-throughput screening applications. Furthermore, human operation can intervene the cells grow, nutrients become depleted and the environment around the cells becomes flooded with metabolic end-products which are toxic and retard cell growth. Therefore a

real-time based on-site microbioreactor monitoring combined with quantitative analysis for cell viability determination is required.

Research shows that different biological cells contain different electrical properties and furthermore, during the past two decades, Bioimpedance has been used to extract biological-related information based on a combination of biological cells electrical properties and invasive detection technology [Zhang et al. 2012; Ghenim et al. 2010; Jiang et al. 2010; Atienza et al. 2005]. By obtaining the electrical impedance, different information relating to the physiology and pathology can be gleaned and this technology is considered to be both accurate and relatively inexpensive [Xu et al. 2015; Chen et al 2011; Yun et al. 2010; Schwarz 1967]. The measuring process often involves the placing of electrodes on the body to submit and receive a small alternating current from which the composition of the internals can be determined [Schwarz 1970]. One of the broadly used methods is Electrical Impedance Tomography technology (EIT), where it injects a small and harmless current into the cell substrate and an electrical impedance distribution inside of a cell substrate is then reconstructed.

3.1.4 Dielectric Relaxation Phenomenon of Biological Cells

The feasibility of utilising bioimpedance spectroscopy technique to monitor cell culture was enabled by the electric properties (known as Dielectric Property) of the biological cell. Most cells are in contact by means of an extracellular matrix or by direct adhesion of one cell to the other constituting different unities [Zhao 2013; Lindstrom and Andersson-Svahn 2010]. The major components of cells are the intracellular fluid, extracellular fluid and cellular membrane. Both extracellular fluid (ECF) and intracellular fluid (ICF) contains ions and are considered electrolytes [Friedman et al. 2010; Yamaguchi 2000; Jordan 1983]. These ions have the capability to move freely and can transport electrical

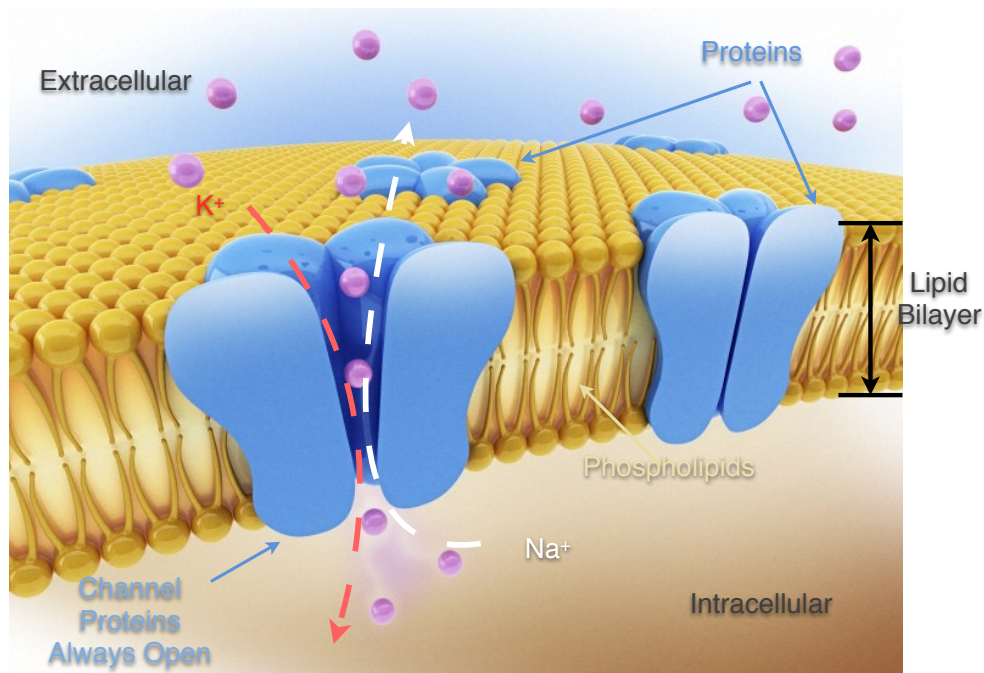


FIGURE 3.5: The diagram illustrated the cell membrane is composed of a phospholipid bilayer and many transmembrane protein including different types of channel proteins that serve as ion channels. Image modified from online. Refers to Weblist.

charge. Thus, both ECF and ICF are treated as resistive elements and, under low frequencies circumstances, ECF and ICF can be regarded as ionic conductors.

3.1.4.1 Electric Property of Biological Membrane

In contrast, cellular membranes are structurally regarded as lipid double layers and full of distributed proteins. Due to the fundamental structure of the membrane, it allows the formation of channels to exchange ions with the exterior as shown in the Figure 3.5. Thus, the membrane effectively acts as a dielectric interface and can be considered as two plates of a capacitor. The Na^+ and Cl^- are reported by literature to be the most common ions contained in the ECF and that potassium ion K^+ exhibits the highest concentration in the ICF. It is with regard to this capacitive nature of cells and tissue that the cellular membrane is considered the most important storage form of chemical energy in the body [Lindner 1983]. Every metabolically active cell of the body can be estimated to have an

electrical potential difference at the cell membrane of about 50-100 *mV* [Grimnes and Martinsen 2008; Jordan 1983; Chernomordik 1983]. A conductor-dielectric-conductor structure is often used to physically model the electric response of cellular substrate by combining the intracellular space, lipid bilayer membrane and extracellular space. The free charges that can move freely on both sides of the cell membrane cause the accumulation of charge, which produces the distinct dielectric properties of the cell if a constant DC electric field is applied. Moreover, an alternating movement of electrons would appear at both sides of cellular channels if an AC electric field is applied which is defined as dielectric relaxation phenomenon. Such phenomenon in the cellular substrate and tissue is the result of the polarisation of several dipoles and the movement of charges [Pliquett et al. 2007]. The dipoles are mainly water polar molecules and the charge carriers are mainly found to be ions in the cellular substrate [Grätzel 2001]. As a result, different relaxation phenomena take place when an excitation current flows through the cellular substrate with various frequencies and has been discussed in Section 3.1.4.5.

3.1.4.2 Dielectric Properties of Biological Cells

In terms of a biological cells structure, both extracellular fluid and intracellular fluid composition and the activity of cell membranes have an impact on dielectric properties which are often referred to as conductivity and permittivity (also known as dielectric constant) respectively. Conductivity is the property of the freely-moving charges inside of the cell substrate (i.e. free movement of various ions and few electrons) in response to the external electric field [Schwan 1963], whereas permittivity is the property of charge that is bounded by biological molecules (i.e. only for those charges that are moving within the degree of molecular lines) in response to the external electric field. To be brief, it is the combination of conductivity and permittivity that collectively represents the biological impedance of organisms, tissue, organs and biological cell substrate when

a weak current is passed through, provided that the passing current is below the threshold that can dysfunction the cellular substrate [Gabriel and Lau 1996; Simpson 1980]. These dielectric properties of cellular substrate are not only determined by the dielectric behaviour of ionic fluids surrounding membrane, but also the polar molecules, proteins in both ICF and ICF space, which are too large to be moved by a constantly supplied alternating electric field. As a result, these particles rotate and align the dipole along the gradient of electrical field instead of migrating and transporting.

3.1.4.3 Conductivity and Permittivity of cells

The literature suggests that different biological cells have different conductivity and permittivity, and that is also related to charges distribution, the externally applied field strength and frequency. In any cell substrate, the charges distribution can never be absolutely even, and therefore conductivity is often considered to be a function of position (scalar). It also partially, but insignificantly, depends on the applied frequency and surrounding temperature. Past research has shown that for some specific type of cells, when temperature changes by 1 °C, the conductivity will change by 1-3 % [Asami 1996]. Under exceptional circumstances, the electrical conductivity of a few types of cells also depend on the direction of the field or current. In such cases, the conductivity becomes a vector rather than a scalar. Thus, it is important to determine the cells type, as their electrical properties may have different responses to the variations in environment. An example of Breast Fat Tissues dielectric property frequency response is shown in Figure 3.6 to illustrate this point. Each polarisation of biological cell is displayed with their individual characteristics, and the dielectric constant is often represented in the complex expression:

$$\varepsilon^* = \varepsilon_\infty + \frac{\varepsilon_0 - \varepsilon_\infty}{1 + j\omega\tau} \quad (3.3)$$

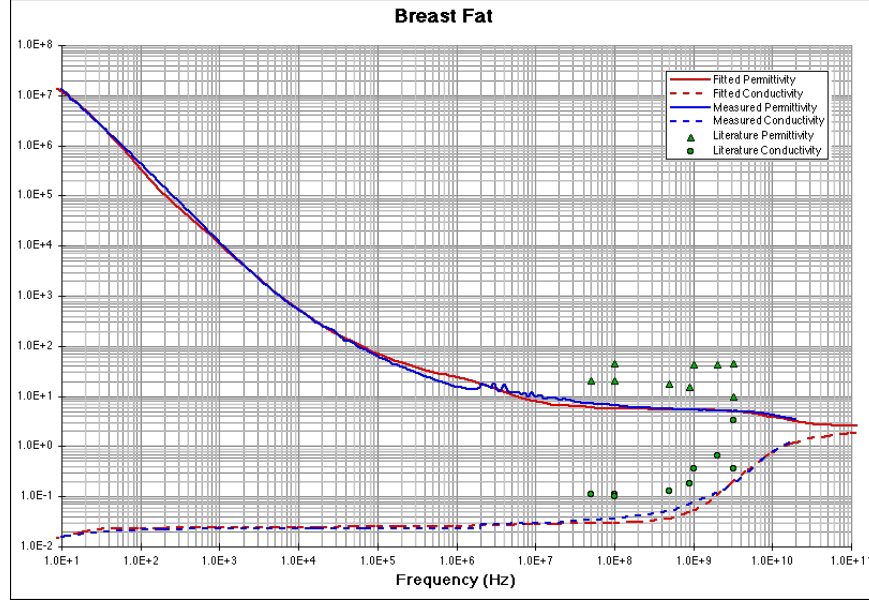


FIGURE 3.6: The illustrated dielectric constant and conductivity of typical biological breast fat tissue as function of frequency. [Gabriel 1996]

In the above equation, ε_{∞} is known as the infinite frequency dielectric constant and can be obtained by measuring with the high-frequency excitation current. In such a case, the polarisation is not taking place in the cell substrate. The ε_0 (also known as static dielectric constant) represents permittivity under low-frequency conditions, in which case, the polarisation is at its strongest. ω is the angular frequency from applied current; τ is the unique relaxation time; and $j = \sqrt{-1}$. The physical explanation for complex dielectric constant is ambiguous and some literature suggests that the real part is directly related to the resistivity of material while the imaginary part reveals whether biomaterial has a capacitive or inductive response. The ratio between these real and imaginary parts indicate the phase lag between electric and magnetic fields. By re-arranging the Eqn 3.3, the real and imaginary parts are obtained as:

$$\varepsilon' = \varepsilon_{\infty} + \frac{\varepsilon_0 - \varepsilon_{\infty}}{1 - \omega^2 \tau^2} \quad (3.4)$$

$$\varepsilon'' = -\frac{\varepsilon_0 - \varepsilon_{\infty} \omega \tau}{1 - \omega^2 \tau^2} \quad (3.5)$$

Thus, the complex expression of dielectric constant of the biological cell can be seen as it generally decreases if the applied signal frequency is increased. However, it is the real part of dielectric constant, which is directly linked with electric field, that is of main interest.

3.1.4.4 Conduction Current and Displacement Current

The passing current is composed of two components, conduction current and displacement current, when there is an AC electric field is applied to the inspected biological substrate. In the electric field, the conduction current, I_c , emerges due to movable ions [Gabriel 1996] inside of the extracellular fluid, which is mainly formed by culture media [Grimnes2000]. Thus, the conduction current is related to both the quantity of ions that are contained by a particular cell substrate and the mobility of the ions themselves. The

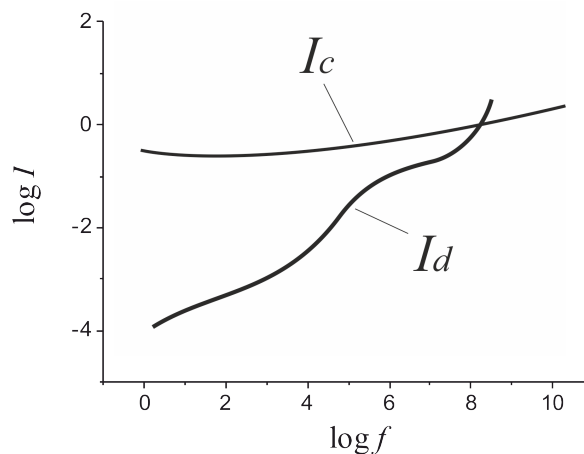


FIGURE 3.7: An illustration of a typical frequency response for conduction current and displacement current [Grimnes 2008].

bounded charge within biological cell contains comprehensive permittivity. In this case, it is known as the displacement current, I_d , which reflects the displacement and the degree of polarisation of bounded charge within the membrane under the electric field. It can be effectively seen as a current that has capability to cross the planar bilayer (membrane). If a frequency-based sinusoidal voltage signal is applied to a cell substrate,

the displacement current (I_d), and the resulting conduction current (I_c), is defined with the following expression and is illustrated in Figure 3.7:

$$I = I_c + I_d \quad (3.6)$$

$$I_c = \sigma V \quad (3.7)$$

$$I_d = \omega \varepsilon_r \varepsilon_o V \quad (3.8)$$

In the above equations, σ is the frequency-dependent conductivity; ω is the angular frequency of sinusoidal voltage signal; ε_o is in the air permittivity and ε_r is the frequency-dependent relative permittivity of cellular substrate.

3.1.4.5 Dielectric Dispersion

Based on the frequency-dependent characteristics, which was discussed in the previous section, both conduction current and displacement current of any cell substrate/tissue changes significantly as the applied frequency increases, in four relaxation windows: α , β , θ and γ as it is shown in Figure 3.6. Each of these steps characterises a type of relaxation that occurs in a specific frequency range and is characteristic for each type of cell and tissue.

The concept of dispersion was firstly introduced by Schwan [Schwan 1957] and dielectric relaxation relation to frequency was derived based on the fact that the cells electric charge cannot keep changing instantaneously with rapidly changing electric fields and, as a result, it forms a time lag.

The α dispersion is produced at the end of low frequency (in the range of 10 to several thousand Hertz). It is thought to be due to a few different mechanisms including counter ion relaxation and frequency dependence of the cell membrane at low frequencies due

to ion channel kinetics. Within this frequency region, when a low-frequency (α dispersion) current is applied to the cell substrate and media, these currents are mainly the conduction currents which are dominated by freely moving ions, and thus only occur in the flow on the cell surface and bypassing the cell membrane. (as illustrated in Figure 3.8). At this point, the basic characteristics of bioimpedance of the cell substrate are mainly resistive.

The β dispersion occurs between the frequency range of 1-10 MHz and is known as structural relaxation. The path of current has a gradual transition in the way that current flows around membrane during low frequency ranges and, to a certain extent, it passes through the cell membrane and intracellular fluid of cells at a mid-high frequency range. Within this mid-high range of β dispersion, the displacement current, I_d , has a more dominant effect. At a mid-high β dispersion range, the possible path of current is illustrated in the middle picture of Figure 3.8. [McAdam 1995]

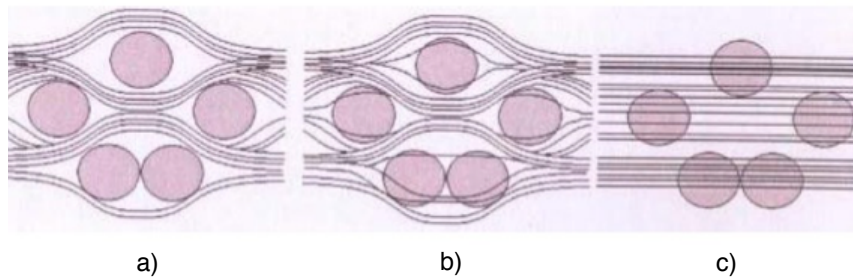


FIGURE 3.8: Illustration of current pathways under different frequency dispersions :
a) α dispersion; b) β dispersion; c) γ dispersion. [McAdam 1995]

The θ and γ dispersion in the microwave band (at 10 GHz and above) is mainly caused by the relaxation process of water molecules (unlike the α and β dispersions). A changing electric field across the material causes energy to be dissipated as molecules attempt to line up the continuously changing electric field and high energy dissipation can cause tissue/cellular substrate damage. This thermal effect of radiation absorption is unlikely

to take place in α and β dispersion, but commonly occurs within θ and γ dispersion region, where a significantly high frequency alternate electric field was applied to the inspected sample. The cellular damage depends primarily on the absorbed energy and the tissue sensitivity. It is also a function of microwave powered density, which depends on the distance from source and its power output, frequency, absorption rate in the given cellular substrate.

Furthermore, it is also reported from past research that cell suspensions also bring the effect in α , β , θ and γ dispersion, but at different characteristic frequencies and magnitudes due to the different cell sizes. However, the main subject-area for dispersion that researchers are interested in for medical application development is in the α , and β , dispersion where most changes between pathological and normal cells take place. For example, when normal cells become cancerous, it leads to changes in their dielectric properties. Some vivo measurements of dielectric properties were taken in order to contrast normal breast and malignant breast cells. As a result, it was found that both the conductivity and dielectric constant in the tumour cell was significantly greater than in the normal cell.

3.1.5 Equivalent Circuit Model for Single Cell

From cells substrate structure perspective, cells are membrane bounded compartments filled with a concentrated solution of chemicals and salts where the membrane maintains an ion concentration gradient between the intracellular and extracellular spaces. It is this electrical gradient that creates an electrical potential difference across the membrane, which is essential to cell survival and is necessary to support movement of oxygen, carbon dioxide, and nutrients.

Due to the electric response in α , β , θ and γ dispersions, it is clear that cell lipid bilayer membrane is devoid of conductive ions but provide ion channels to facilitate

the transcellular communication, which can be modelled as a parallel combination of resistor and capacitor. On the other hand, the free moving ions richly contained in the extracellular and intracellular fluid majorly indicate the conductivity and thus determine the conductance of cell substrate. Thus the biological cell and its extracellular matrix can be considered equivalent to an electrical network,

3.1.5.1 Geometrical Model

The impedance of a biological cell substrate, known as bioimpedance, Z , of cell substrate is defined as the combination of conductance and capacitance and the relationship is as follows:

$$Z = R + jX_c = \frac{1}{Y} = \frac{1}{S + j\omega C} \quad (3.9)$$

$$S = K\sigma(\omega); \quad C = K\varepsilon_0\varepsilon_r(\omega); \quad K = A/l; \quad (3.10)$$

Where Z is bioimpedance of cell substrate; Y is the inverse of impedance which is known as admittance; S is the equivalent conductance, which is dependent on excited frequency; C represents the equivalent capacitance, which is dependent on excited frequency; K is the geometric constant which is dependent on the cross-section area and length of measured cell substrate. The very first effective model, named Frick Model, was introduced in 1924 which is shown in Figure 3.9.

3.1.5.2 Fricke Equivalent Circuit Model

The cell membrane capacitance is significantly higher than its trans-membrane ionic channel resistance in all frequency range, in which case, R_m is almost negligible and can be regarded as a short-circuit path. The equivalent electrical circuit then compressed as part c). In this model, the capacitor, C , exists in the biological cell and is frequency, ω , dependent. During the low frequency range, the membrane functions as an insulator

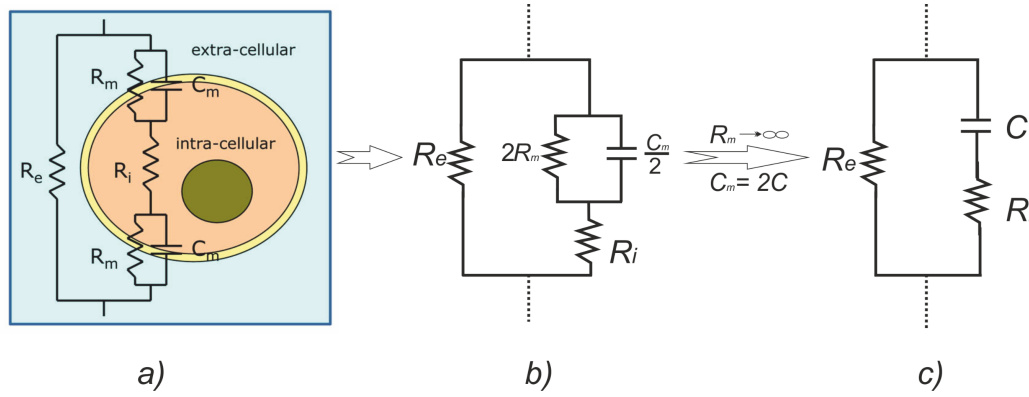


FIGURE 3.9: Equivalent circuit of a cell where R_e is the extracellular fluid resistance, R_i is the intracellular fluid resistance, R_m is the trans-membrane ionic channel resistance and C_m represents the cell membrane capacitance

so that current cannot penetrate the cell, and as a result only extracellular fluid resistance, R_e , can be measured. Whereas at high frequencies, the current begins to flow through both intra and extracellular fluids and the overall impedance is represented as R_i in parallel with R_e . The impedance of a cell follows the Fricke model, which can be expressed as follows:

$$Z = \frac{R_e(1 + R_i j\omega C)}{1 + j\omega C(R_i + R_e)} \quad (3.11)$$

3.1.5.3 Cole Equivalent Circuit Model

This Fricke model for biological cells was examined by Kanai et al in 1983 and Jaffrin et al. in 1997 it was concluded to be only suitable for blood cells since haematocyte only contains one dominant cell species [Jaffrin 1997]. Alternatively, Kenneth S. Cole proposed an empirical equation to describe tissue impedance followed with an improved version known as $Cole_z$ equation:

$$Z = R_\infty + \frac{R_0 - R_\infty}{[1 + (\frac{j\omega_c}{\omega_0})^\alpha]} \quad (3.12)$$

R_0 represents the impedance of a biological cell under direct current (or low frequency) conditions (in this case, the cells bioimpedance is regarded as a resistance); R_∞ represents impedance under high-frequency conditions; ω_c is the angular frequency; ω_0 is the cells characteristic frequency (which means that, at this frequency, the imaginary part of cells bioimpedance reaches its maximum value); and α represents the distribution parameter (with a value between 0 and 1). When the distribution parameter, α , is set to be equal to 1; Eqn 3.12 is re-arranged as follows:

$$Z = R_\infty + \frac{R_0 - R_\infty}{[1 + (\frac{j\omega_c}{\omega_0})]} \quad (3.13)$$

If resistances are set as $R_2=R_\infty$, $R_1=R_0-R_\infty$, then the *Cole* equation can be modelled by a simple RC equivalent circuit system as shown in Figure 3.10.

In Figure 3.10, the series connected resistor R_2 represents the resistance of the intracellular fluid; the parallel connected resistor R_1 represents the cell membrane resistance; and capacitor C represents the capacitance that is formed by the cell membrane. At high frequencies, due to the functionality that cell membrane capacitance contains, it causes the cell membrane resistance to be bypassed, and the cell is rendered as a pure high-resistance (with value of $R_2=R_\infty$); while at low frequency conditions, the membrane capacitance C can be regarded as an open circuit, in which case the bioimpedance of the cell can be regarded as a summation of R_0+R_∞ that are series connected.

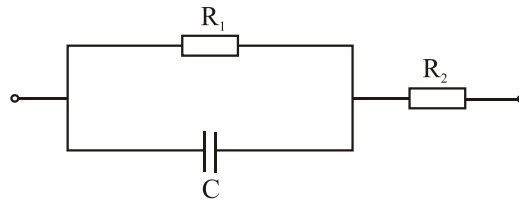


FIGURE 3.10: Equivalent circuit of simplified Cole model for tissue impedance systems

In conclusion, both circuits includes Fricke circuit and Cole equivalent circuit, are complementary models and a choice for appropriate model has to be made when measured data is to be analysed and presented. The choice is based on the presumption of the actual physical arrangement in the electrode data acquisition system to be modelled and hence it is decided by the measurement setup: by choosing constant amplitude voltage or current mode from the signal generator or by choosing parallel or series coupling in the bridge arm.

3.1.6 Development of BIS Measurement

The electrical bioimpedance is a non-invasive, quantitative, label free and real-time physiological monitoring modality. It generates signals smaller than thermal noise at room temperature (≤ 25 mV). Classical works in the field of bioimpedance were contributed by Kenneth Cole, Peter Debye, Robert Cole and Herman Schwann. Kenneth Cole mainly focused on the investigation of electrical impedance of squid axon [Cole 1939], and dielectric dispersion range [Cole 1928] and Herman Schwann facilitated the understanding of electrical characterisation of cellular substrate and tissue and more importantly the electrode polarisation [Simpson 1980]. Kubicek et al. (1970) and Mount (1974) took the in-situ measurements using impedance cardiac stroke volume followed with bacterial growth monitoring and anchorage dependent cell culture where the published results were re-confirmed by Giaever et al in 1984. In 1987, Professor Sakamoto established an experiment based on the above model, using a 16 bar electrode, 50 kHz, 1 mA and the constant current excitation measurement system [Kanai and Sakamoto 1987]. The technique was known as Single-Frequency Bioimpedance Analysis (SF-BIA). With the development of computers for measurement and control applications, biomedical measurements opened up a new path as the electrical properties of cells can be more accurately and efficiently measured. Bioimpedance techniques have been become broadly utilised to study cellular substrate, organ and tissue in the body [Dijkstra et al.

1993]. Much of the early bioimpedance work is summarised in the review by Jossinet (2005) and McAdams (1995).

However, the literature shows the single frequency based experiment cannot be reproduced and many tests on the conclusions of BIA have not brought about a broad consensus [Jossinet 2005]. So in recent years, the trend of research on the bioimpedance measurement is changing from one frequency to sweep-frequency which is known as bioimpedance Spectroscopy (BIS). Mathematically, it is more complicated to predict R_0 and R_∞ for BIS (by utilising Cole-Cole plots and Hanai equations). Furthermore, despite that the BIS method has been proved to be accurate for healthy populations, the BIS method can potentially only be exhausted if the data is interpreted with sufficient algorithms that include a valid fluid distribution model and reliable data fitting.

Over last decade, several sensing and monitoring technique via BIS were proposed by several institutes and universities. The pioneering work of Giaever and Keese for using BIS to monitor anchorage dependent cell culture has led to many applications of Electrical Cell Substrate Impedance Sensing (ECIS) as a viable tool for physiological analysis [Muller et al. 2011; Hug et al. 2003; Keese et al 2000]. ECIS was fabricated based on a two-electrodes impedance measurement system. A 250 μm diameter working electrode and a forty times larger counter electrode was used to measure the complex voltage with 1 μA current. It is SF-BIA system and only recorded at a single frequency of 4 kHz . As a result, real-time monitoring of cell layer and parameter characteristics are not available in the ECIS system. Borkholder et al. from Stanford University in 2003 demonstrated a 40 electrode array sensor for characterisation of the effects of pharmaceutical compounds on cell membrane. The so-called cell-based biosensor is categorised into voltage-drive pattern where 100 mV is applied to the cellular substrate which is well over the limit of linearity in bioimpedance spectroscopy (25 mV at room temperature) [Barsoukov 2005]. The published results provide non-linear characteristics and parameter extraction was not implemented. A similar multi-electrode array system was proposed by Huang et al.

(2005) to study cellular behaviour where data is acquired via three-electrode molar system and is lack of automation. A Real-Time Cell Electronic Sensing (RT-CESTM) was manufactured by ACEA Biosciences, which is a cell culture monitoring device based on sensor arrays. There is an improvement in integrability between measurement system and standard microplates but the electrode effect was not discussed and more importantly it can only operate for SF-BIA rather than BIS. The four-electrode tetrapolar system was initially implemented by the World Precision Instrument (WPI) which is world leader in Trans Endothelial Electrical Resistance measurements (TEER) [Ivorna 2003]. The TEER device consists of a resistance meter and four-electrode chamber. Regarding material, the current excitation electrodes were made of silver-silver chloride which is not suitable for cell monitoring. Furthermore all of abovementioned techniques introduced in early twenty-first century were large spaced and lacked customisation.

In more recent years, there is a clear tendency to reduce the size of BIS systems to provide an in-situ monitoring spectroscopy of cellular substrate. In particular, the feasibility to monitor the viability of cell culture through interdigitated electrodes [Bhansali et al. 2015; Lu et al. 2013; Thakun et al. 2012; Rahman et al 2008] was reported. Results revealed the potential correlation between measured bioimpedance magnitude and cell viability. The concept of utilising existing instrument on a PDMS microbio-reactor platform was introduced by Lei et al. (2012) and Lin et al. (2011) where the electrodes were horizontally inserted into culture chamber and directly in contact with cell substrate. The reduced size for BIS system was achieved by utilising the existing impedance measurement instruments, which can be divided into two general categories, namely LCR-meters and impedance analysers. Both LCR meter and Impedance Analyzer can only support two-electrodes or three-electrodes setup which is not suitable for impedance measurement for cellular substrate, which will be discussed in the next section.

Moreover, in order to fit the microbio-reactor in-situ monitoring purpose, there is a clear

demand to miniaturise the BIS to be fully compatible with PDMS material as well as become portable for necessary cell culture implementation outside of the incubator. The miniaturising of BIS system was enabled by the enhancement of functionality of electronic chips and a number of design and fabrication were implemented over the last three years. Though the accuracy of BIS performance over low frequency range was intensively discussed, but the evaluation of fabricated for cell culture was generally ignored.

3.1.7 Electrode Interracial Effect for BIS Measurement

It is noticed here that the majority of previous publications in the bioimpedance field have treated electrodes as merely tools to operate under such setups. However, the risk of the interface harming the cell during the culture period has been overlooked. Therefore, there is a crucial need to discuss the stability and sustainability of electrodes performance through long term static cell culture.

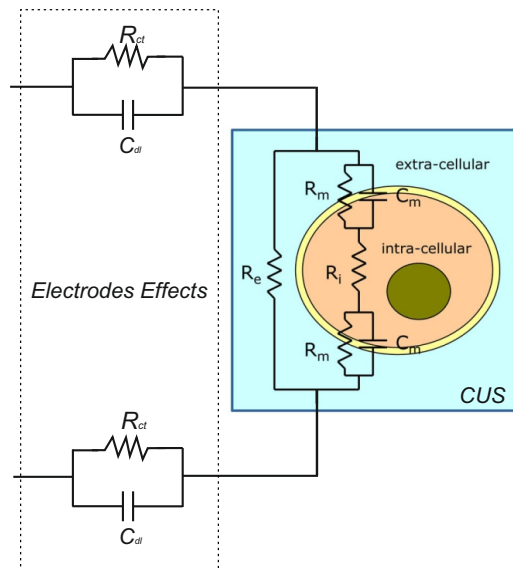


FIGURE 3.11: Illustration for electrode interface effect during measurment

On the other hand, if the excited current continues to increase in amplitude, provided the frequency of excitation current remains unchanged, the applied stimulus current is high enough to heat up the cell substrate and will lead to major biological effects. Some types of cells are more sensitive to either amplitude or frequency of excited signal. Therefore, these show that there is a potential risk when the cell is connected to an external current and both the amplitude and frequency that are injected into the cell substrate must first be deemed to be adequately secure. It is important to not to confuse the non-linear impedance response of cellular substrate and the non-linear impedance response of electrode-electrolyte interface that is often categorised as electrode kinetics.

There exists two types of processes on the surface of electrodes, namely Faradaic and non-Faradaic processes. Both faradaic and non-faradaic processes exist as long as the conductive electrode is inserted into biological substrate. In Faradaic processes, charges are transferred across the metal electrolyte interface. However, adsorption and desorption can take place if the potential across electrodes are changing and it is termed as non-Faradaic process. Despite the fact that charge cannot penetrate through the interface in non-Faradaic process, the existence of transient current inside of the interface cannot be avoided due to continuous charging and discharging at the interface. The theory of the electrode effect, known as electrical double layer, was explained and discussed in details in Section 3.1.2.1 earlier in this Chapter and consequently the combinational model of Chapman and Stern theory are applied under such circumstances. As a result, the electrode electrolyte interface itself gives rise to complex bioimpedance and the equivalent model circuit can be seen in Figure 3.11.

More importantly, it is genuinely true for current-carrying electrode to form an interface with cellular substrate where irreversible oxidation or reduction process may take place. Thus, probing the interference of potential redox processes with cellular substrate on different electrode materials in an oxygen permeable microenvironment, i.e PDMS, not only aids development of more suitable microenvironment for cell culture, but also

promotes the optimisation to minimise or at least control the influence of the interface on measured bioimpedance data. It is therefore required to investigate the interface between electrodes of different materials and cellular substrate, by utilising the adequate model via three-electrode monopolar data acquisition system.

The direct electrochemical investigation of electrodes and their interaction with biological solutions is often hindered by 'irreversibility' of the electrode surface, hence the voltammetric responses of the cathodic and anodic processes have to be analysed separately.

Capacitive leakage is one of the main sources of error in reported bioimpedance measurements [Buendia et al. 2010]. It is possible that current will flow in the cellular substrate without flowing through the sensing electrodes or the output of impedance of the current source in a standard electrodes data acquisition setup. Furthermore, if the current does not flow through the injecting electrodes under standard measurement construction, the crosstalking effect will be created. In such case, the current will flow through a crosstalk capacitance, which is known as inter-electrode capacitance, C_{ie} , instead of through electrodes interface due to the high electrode impedance value. The additional errors, for estimated value of cellular bioimpedance, will therefore be introduced in the data acquisition system since the abovementioned crosstalk effect will create an additional voltage drop that is sensed by a differential amplifier.

3.1.8 BIS Data Acquisition Systems

In the existing bioimpedance spectroscopy (BIS) data acquisition system there are three basic forms: current driven pattern, voltage driven pattern and the more recent induced current pattern. Among those three patterns, current driven pattern [Figure 3.12] has lower requirements for data acquisition setup but there are unidentified limits for injecting current magnitude and frequency under current driven pattern. The limits were also

reported to be linked to cellular substrate volume and monitored cell types [Grimnes et al 2011; Grimnes and Martinsen 2007]. Other effects include electrode polarisation effect, capacitive and crosstalking effect, which also influences measurement setup and its accuracy [Kalvoy et al 2009].

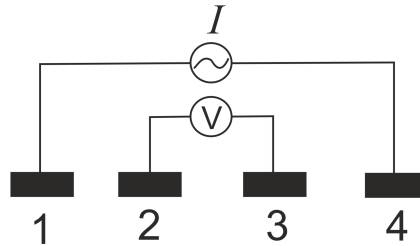


FIGURE 3.12: Illustration for Current Driven pattern measurement setup

It is clear that large currents are favourable to increase the voltage drop for potential measurements and, consequently, to maximise the signal-to-noise ratio during BIS monitoring measurement. However, two facts that limit the allowable current intensity include non-linear behaviour of living cellular substrate and safety issue. In the cellular substrate, the voltage and current relation has a linear characteristic only if the applied electrical field does not exceed the non-linear frequency threshold [Challis et al. 2005; Weaver and Astuman 1990; Pethig et al 1987]. The closing and opening of ionic channels on the cell membrane will be polarised if the potential difference between the intra and extracellular media is around tens of mV (i.e about 10 kV/mm) which is caused by a superimposed electric field. The protein structure of the cellular membrane that is responsible for the transport of specific ions will change its conformation and hence change the membranes conductivity. It is therefore to make sure that the excited current frequency does not exceed the specific type of cellular membranes polarisation threshold. An extreme case is that of human skin which would be nonlinear if current density became as low as 100 uA/cm^2 at 100 kHz [Ollmar 1998]. Thus, a number of parameters that could potentially contribute to the limitations of data acquisition system should

be taken into careful consideration, including current density, applied voltage, electric field, cell type, shapes and positions of electrodes.

3.1.8.1 Two-Electrodes Biopolar Setup

In fact, literature shows that many experiments use two-electrodes systems for bioimpedance measurement where the validation of the experimental results can be debatable. This is because for two-electrodes configuration the injecting leads and the signal sensing leads are connected to the same pair of electrodes and hence causes the polarisation on the electrode surface. The methods can be used as a rough estimation if and only if the impedance of electrodes interface, Z_{ep} , is significantly lower than the impedance of the cellular under studies, Z_{CUS} , since the measured impedance $Z_{measure}$ is the sum of Z_{cus} with two Z_{eps} . The electrode-electrolyte interface impedance is expected to be much higher than the impedance under test, especially at low frequency range, and are too instable and unpredictable to think of a mathematical correction of the measurement. Therefore, the repeatability and the reproducibility of the measurements can be seriously compromised.

3.1.8.2 Four-Electrodes Tetrapolar Setup

Most publications in either single cell analysis or cellular substrate determination relies on existing two electrodes system where the impedance effect of bipolar electrodes bring significant distortion in bioimpedance measuring, whereas three-electrodes monopolar setup, as discussed in Chapter 3.1.2.2, focuses on the material interactions instead of bioinformatics. The Cyclic Voltammetry methodology was guaranteed by utilizing the electronic analogue circuitry which was discussed earlier and it has been intensively applied in the precise detection for specific protein-protein interaction and enzymes analysis. However, both setups are not suitable to be employed for cellular substrate

analysis since the impedance of electrodes, Z_{cus} , inevitably brought a large effect to the monitored system, causing the obtained data to fluctuate. The distal volume segment of the current part became the area of interest in bioimpedance analysis, and the effect of the zones proximal to the current carrying electrodes is expected to be eliminated or at least reduces to the controllable level.

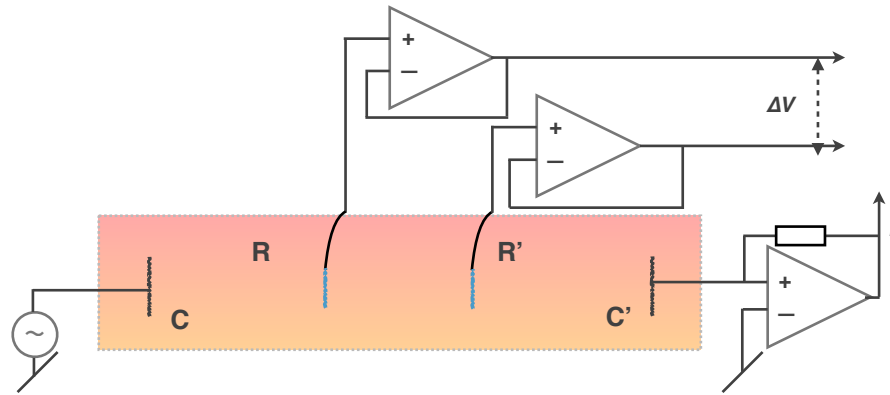


FIGURE 3.13: Demonstration of Tetrapolar electrode setup for bioimpedance measurement in cellular substrate

The four-electrodes methods, also referred as Tetrapolar or Kevin method [Grimnes and Martinsen 2007] where four probes are divided into two pairs and a setup for in-vitro measurement is illustrated in Figure 3.13. The outer pair of electrodes are current stimulation electrodes; whereas the inner electrodes are potential electrodes as illustrated in Figure 3.14. The two recording electrodes ensure that only the current path segments between these electrodes contribute to the results. The sensitivity is proportional to the local current density in the measured volume segment. The diagram is used as demonstration here and the full technique details and derivative refers to Appendix C.

The measured segment is determined by the position of the two potential recording electrodes R and R' respectively. It is generally assumed that no current is flowing in the electrodes leads, thus polarization cannot be triggered by external stimulation. However, past research suggested that the ionic current flow pattern in the cellular substrate can be interrupted if electrodes are directly in contact with the measuring cells. Thus it

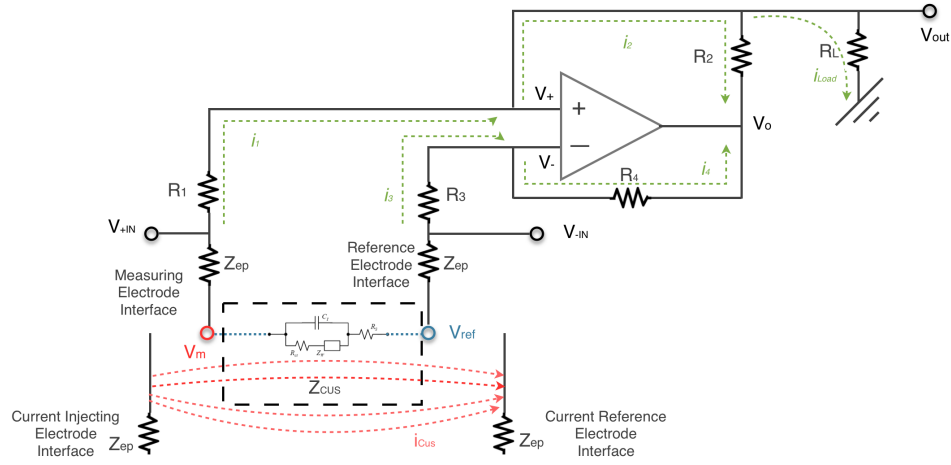


FIGURE 3.14: An equivalent circuit model for Tetrapolar Data Acquisition System

is advised to avoid embedding the electrodes at the bottom surface of PDMS culture chamber during fabrication via soft lithography.

As a result, in contrast with the two electrode methodology and three electrodes, no current flows through the potential measuring electrodes and the injected current flows completely through the biological cellular sample and hence the detected potential difference across the sample is directly proportional to the electric response of the cellular substrate. As a result, the influence of the inaccuracy by electrode interface is significantly reduced. It is noted that the four-electrode methodology is only applied when electric field distribution is uniform, in other words the two sets of electrodes are either on the same plane or parallel planes and the current is perpendicular to the plane.

3.1.8.3 Sixteen-Electrodes Setup (Electrical Impedance Tomography)

More advanced data acquisition setup was also examined during past research and electrical Impedance Tomography (EIT) is known as one of the most popular data acquisition systems for bioimpedance measurement. In principle, the two selected current excitation electrodes send a safe current through the cellular substrate and then measure the corresponding substrate for biological information so to reconstruct an image

which shows the impedance distribution within the substrate/organ and changes in the distribution [Holder 2005]. Figure 3.15 is a diagram demonstrating the principles of the collecting methods of EIT technology. This methodology is also called the Neighbouring Method, the excitation current is injected through two adjacent electrodes resulting in a sensitive field. The voltage between the adjacent electrode-pairs is measured in turn. Then the excitation current is applied to the next electrode-pair while the voltage is measured on the other electrode-pairs that are not injected with current. The system has mainly been employed in the biomedical image reconstruction applications, but also gaining popularity for bioimpedance analysis (BIA) and spectroscopy (BIS) measurement. It is noted in this chapter that the sixteen-electrodes based bioimpedance monitoring system for EIT was physically realised. Nevertheless, the evaluation of system with different types of cells during culturing period is outside the scope of this thesis.

FIGURE 3.15: Illustration for 16 Electrodes based EIT system

In this Chapter, a heating-controlled integrated and portable microbioreactor platform for DLD-1 cancer cell viability determination is designed and fabricated by applying the electrical properties of the cells combined with the simplified EIT technique to measure cells' bioimpedance and thus determine the viability. The device mainly consists of five embedded parts which include a cell culture chamber, known as microbioreactor; an EAVL-AD5933EBZ chip, a commercially available electrical-impedance analyser chip;

a multi-electrode based data acquisition system; a 8×16 switching array unit, an analogue front end circuitry. The reliability of proposed analogue front-end circuitry for providing AC current with small magnitude ($70 \mu\text{A}$) was designed and examined. The feasibility of using 8×16 analogue switching array as a replacement for conventional switching relays was assessed within systematic operations. Furthermore, the electrode polarisation impedance of microelectrodes is significant and hence a number of different materials were tested via Cyclic Voltammetry and evaluated to be used as microelectrode for bioimpedance monitoring purposes in long term cell culture. An additional ITO-film based heating system was provided and integrated with proposed miniaturising BIS monitoring outside of incubators to ensure the dielectric properties of cellular substrate will not be affected by change of physiochemical culture environment since the past research suggested that for temperature changes of 1°C , the conductivity will change by 1-3 %. Cancer cell line DLD-1 was loaded into the microbioreactor for viability test for 24 hours. A reasonable linear correlation between haemocytometer counted cell viability and its bioimpedance was illustrated. The results show that the proposed technique provides an alternative and efficient method to monitor the cells viability in an in-vitro physicochemical environment during cell culture process compared with the conventional cellular analysis techniques, and this may have a potential application to control the cell differentiation process. Additionally, the system will have immediate applications for the 3D cell culture and the presented mechanism is also promising to be integrated with a multi-channel based perfusion system for high throughput cellular analysis.

3.2 Material and Methods

3.2.1 System Design Principle

The microbioreactor platform was designed for providing portable BIS measurement during the cell culture process without affecting the cellular behaviours and to offer additional information alongside microscopic observations. This platform is achieved by introducing a new electrical impedance analyser chip, which can be integrated with analogue front-end circuit to operate Bioimpedance Spectroscopy combined with a simplified EIT data acquisition system. The Reduced EIT data acquisition system refers to the system when the reconstruct imaging processing is disabled as it is irrelevant to BIS in-situ measurement.

The microfluidic platform contains four embedded sections which include a cell culture chamber, an EAVL-AD5933EBZ chip, a commercially available portable electrical-impedance analyser chip; a multi-electrode based data collection system (reduced EIT system) and an analogue front-end circuitry. The illustration of the platform is shown in Figure 3.16.

Due to the different need of data acquisition system to fulfil the different biological inspections, both tetrapolar and EIT-based data acquisition were considered during the system design. The tetrapolar data acquisition system was achieved by introducing four four-electrodes into each single culture chamber and each electrode is made of selected material with a 0.35 mm diameter rod shape with 15 mm length. Only 4 mm of electrodes length are in contact with culture medium but it is 0.4 mm above the bottom surface of PDMS microbioreactor chamber as electrodes shouldn't directly contact with cells in a monolayer formation, and it is illustrated in figure c). Their electrical contact to the external measurement equipment was post-embedded in the PDMS after the fabrication of the microbioreactor took place and were sterilised under 135°C for one

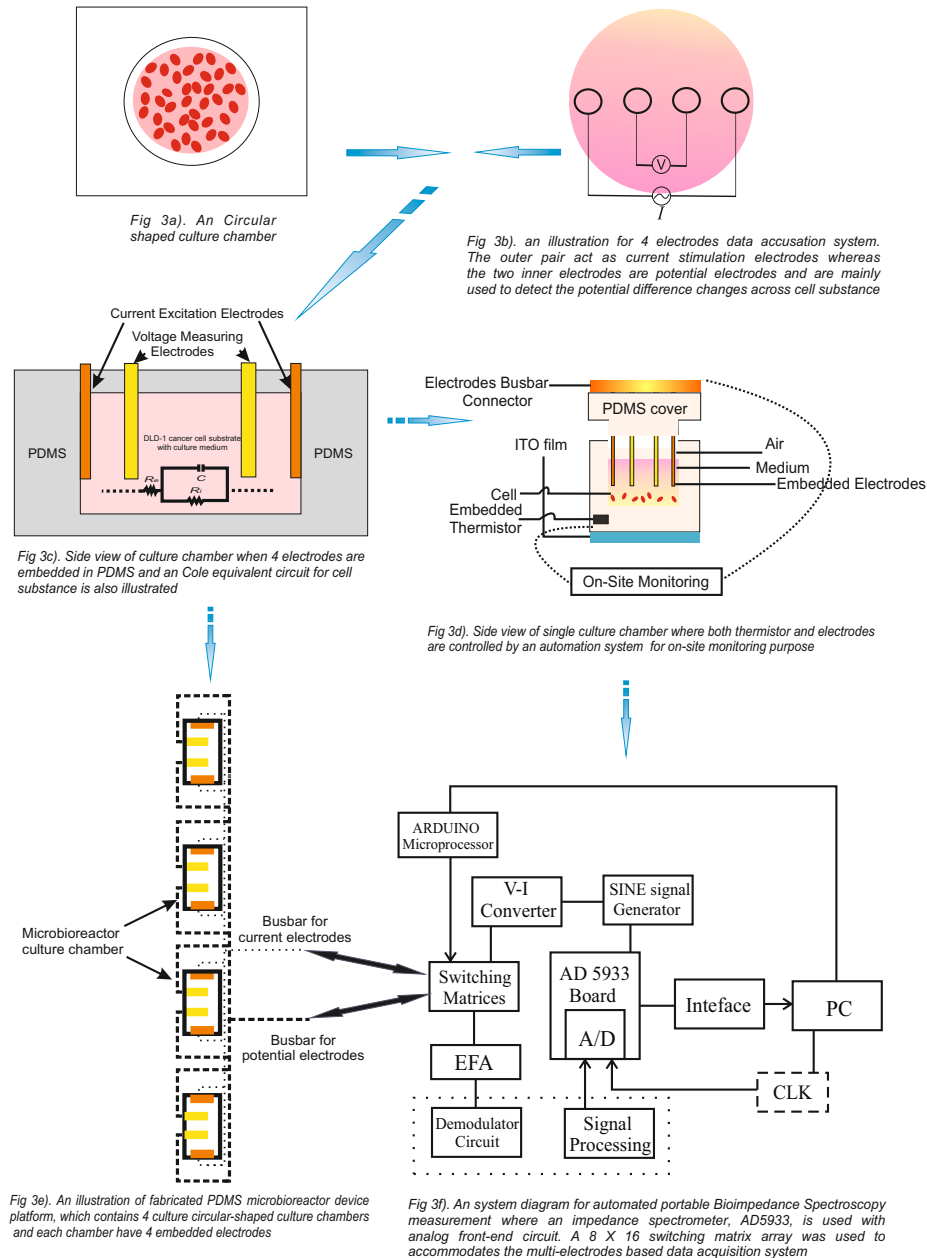


FIGURE 3.16: Top level design of the portable Bioimpedance measuring system. A) Circular shaped culture chamber; b) A top-view illustration for 4 electrodes data acquisition system. The outer pair act as current stimulation electrodes whereas the two inner electrodes are potential electrodes and are mainly used to detect the potential difference changes across cell substrate; c) Side view of the culture chamber when 4 electrodes are embedded in PDMS; d) side view of single culture chamber where both thermistor and electrodes are controlled by an automation system for in-situ monitoring purpose; e) An illustration of fabricated PDMS microfluidic device platform; f) The schematic diagram for automated portable BIS measurement

and a half hours. The other type of data acquisition system obeys the simplified EIT system which is illustrated in Figure 3.17.

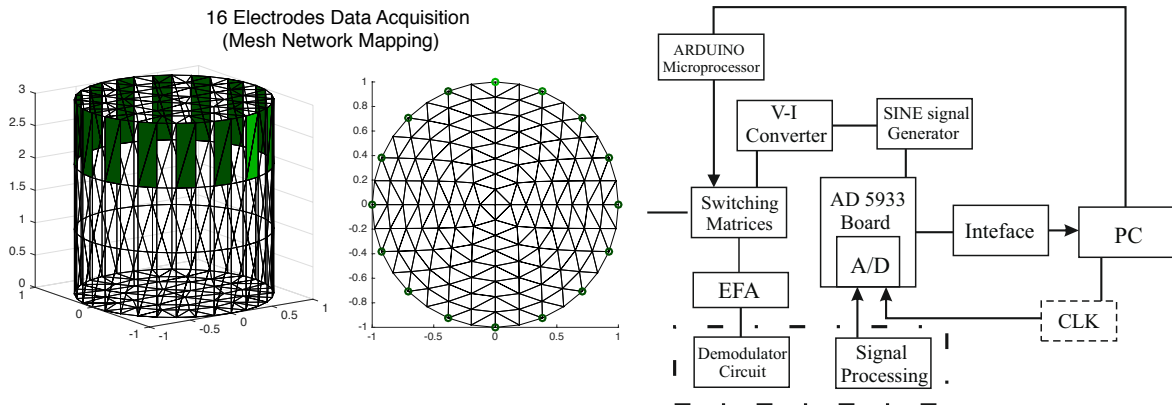


FIGURE 3.17: Illustration of fabricated 16 Electrodes based EIT system

It was part of the proposal to design and fabricate a BIS system with high flexibility and compatibility to different data acquisition systems and hence to meet different biological inspection needs. Thus, it is noted that both types of data acquisition systems were designed to cooperate and to be integrated with the same data processing and analysing system without the need of changing instrument setup.

3.2.2 Fabrication of the Microbioreactor

The culture chamber is circular-shaped, where dimension of chamber is 15.58 *mm* diameter and 8.0 *mm* depth, for accommodating the cellular substrate. It is made of polydimethylsiloxane (PDMS) material and fabricated by soft lithography. The process is briefly described in Chapter 2.2.1. Instead of 10:0.85 ratios between prepolymer PDMS and its curing agent, a new ratio of 10:1.05 was applied to improve the PDMS material stiffness after solidification under this circumstance. The liquid-state PDMS and curing agent was fully mixed by external mechanical force and were poured on the

master for the fabrication of both the cover and the chamber unit. Meanwhile thermistors for the heating system for each chamber were embedded into the liquid-state PDMS with 1.5 *mm* distance to the bottom surface of microbioreactor and 1 *mm* horizontally distant away from culture chamber. The polymer with thermistors were then de-aerated under vacuum to remove all air bubbles created during mixing followed with a solidification process. After baking for solidification, both PDMS layers are peeled off from the master. The electrodes with selected material were sterilised in autoclave equipment (Derma Contour, Germany) under 135 °C continuously for one and a half hours and were embedded into the system in a sterilised environment.

3.2.3 Electrodes Characterisation

The concept of biocompatibility and non-destructive characteristics of electrodes material to cellular substrate over long term cell culture was assessed by Cyclic Voltammetry via three electrode monopolar systems.

An AutoLab PGSTAT20 computer controlled potentiostat (EcoChemie, Utrecht, The Netherlands) was used to perform electrochemical measurements. A three-electrode configuration was used for electrochemical measurements under static aqueous solution environment in a Faraday cage. The cyclic voltammetry operation was implemented in the experiment where a dc voltage of staircase characteristics was applied. The voltage was swept at a predetermined voltage level from -1.0 V to +1.4 V. The sweep starts at +0.3 V and scans cathodically towards -1.0 V. It then reversed and scanned anodically to +1.4 V and the cycle ends at the + 0.3 V. Such a voltammetric cycle repeats sequentially for 3 times during each measurement. Four different materials namely silver wire (99.99 %, 0.35 *mm* diameter, GoodFellow, Cambridge, UK), gold wire (99.99%, 0.35 *mm* diameter, GoodFellow), graphite rod (99.999%, 0.35 *mm* diameter, GoodFellow) and platinum wire (99.99 %, 0.35 *mm* diameter, GoodFellow) were used as working

electrodes respectively whereas the reference electrode and the counter electrodes were chosen to be platinum throughout all cyclic voltammetric measurements. The working electrodes consist of exactly the same electroactive surface area, i.e. 0.157 cm^2 . Regeneration of silver surface was achieved by piling with coarse paper -1000. In comparison, both gold and platinum wires were regenerated with Butane torch at 1200 degree and graphite rod was cleaned in an autoclave. All electrodes were then rinsed with ethanol and phosphate buffer solution prior to incubation in culture medium.

Three aqueous conditions are probed within the electrode characterisation section. Each type of working electrode was investigated in $385\text{ }\mu\text{l}$ freshly prepared cellular culture medium in PDMS microbioreactor culture chamber. Additionally, except silver, the other three types of working electrodes were used in $385\text{ }\mu\text{l}$ fresh culture medium solution with unsuspended H1299 cancer cell with 10^7 cell density in PDMS culture chamber. Further comparative experiments were implemented for graphite and platinum with 5 days statically cultured cell sample in a fully metabolised culture medium. All measurements were carried out under sterile conditions. Data was acquired and analysed through use of a commercially available software package Nova V.1.11 (Metrohm).

3.2.4 Circuitry Design Principle and Analogue Front End Investigation

The critical issue in current-drive system is to construct a PC controllable precise micro-levelled current source over a large range of frequency spectrum. The stability and reliability in a portable device to provide such small valued current has been a well-known problem and hinder the potential application in clinical and medical fields. On the other hand, it was found that an A/D based high precision impedance converter chip, AD5933EVAL, was able to offer the ability to measure the impedance directly by sending constant voltage and receiving measured current. The composed block of

AD5933 is shown in Figure 3.18.

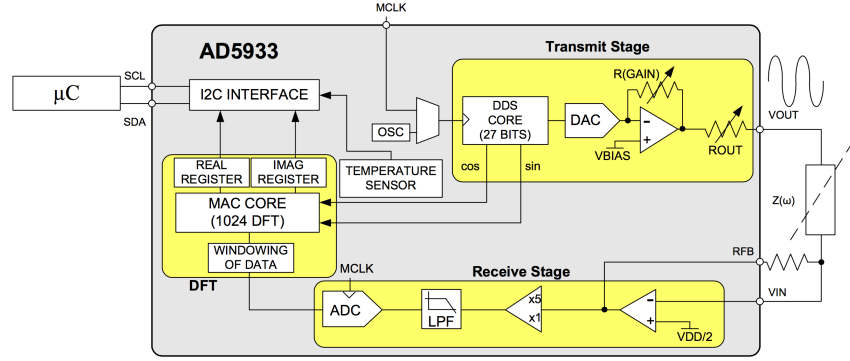


FIGURE 3.18: Illustration of composed block of AD5933 chip. Image from Manufacturer datasheet, refers to Website-List.

$$\frac{V_{out}(\omega)}{V_{in}(\omega)} = \frac{Signal_{referential}}{Signal_{measurement}} \quad (3.14)$$

In principle, the measuring load is connected with V_{out} and V_{in} pins and, when a measurement is taking place, the output excitation signal with a particular frequency will then be applied to the load. On the other hand, the current that flows through the impedance of measuring load is acquired by the response channel. In the response channel, the current sensing is composed of a current-to-voltage amplifier with a programmable gain amplifier (PGA) and further with an anti-aliasing filter and an ADC.

As illustrated in Figure 3.16 C), the Analogue Devices newly released system-on-chip fully integrated electrical impedance analyser is used in the measurement system, which allows the implementation of minimum-size instrumentation for bioimpedance measurements instead of using large-sized professional impedance spectrometer.

Nevertheless, despite the high precision impedance measurement of AD5933EVAL chip, it was not intended to be used for bioimpedance measurement due to five main factors: (i) it is a voltage driven system and cannot directly control the injecting current in which case the injecting current can potentially be higher than the limits of current in cellular

substrate which are set by strict regulations [Schmidt 2005 IEC-60601-1 2005]; (ii) it has large output impedance and hence can only measure the impedance that is not lower than 1 kOhms where as many of biological tissue can be significantly lower than thousands of Ohms (iii) the system itself only supports a two-electrode bipolar data acquisition system which is not suitable for most applications of bio-electrical impedance and needs re-arrangement to adapt to the four-electrode and sixteen electrodes strategy; (iv) the voltage output contains a DC excitation which will cause safety concerns because when a dc voltage is placed across the electrodes and the Cellular-substrate Under Study (CUS) it may cause some hazard for cellular substrate; (v) with regard to of the upper limit frequency from the AD5933EVAL data sheet, the chip can provide precise impedance measurements up to a maximum frequency of 100 kHz . It is unclear whether the limitations for frequency range would affect the accuracy for BIS measurement for various types of cellular substrate. Thus the reliability and accuracy needs to be investigated for portable BIS measurement by combining the AD5933EVAL and corresponding analogue circuit.

Thus an analogue front-end (AEF) circuitry investigation is essentially needed in the designing system. In short, it is suitable to consider the AFE as a combination of one high pass filter with two V2CC, one in the direction from AD5933 to CUS and another from CUS to AD5933. The first V2CC accepts the output (voltage) signal, from the EVAL-AD5933EBZ and the value of this output voltage signal, V_{out} , is directly related to which operation mode is chosen. As the V_{out} contains a DC bias component, a high pass filter is added to remove the bias component before it reaches V2CC and causes operational instability. Once the V2CC is excited by the AC V_{out} , it then acts as a voltage-controlled current source (VCCS) and generates a controlled AC current, (I_{out}), into the CUS, and such controlled current causes the voltage across CUS to drop, which is then sensed by the second V2CC. Similarly, the second V2CC will generate AC output current and a DC component is added which is equivalent to the DC bias which was

For true V2CC, I_{out} needs to be independent of V_L and R_o requires to have significantly large value. The magnitude of R_o in practical op-amp has been discussed later in this chapter. By assuming employing true V2CC, the gain factor of the system can be expressed as following.

$$\frac{V_{out}(\omega)}{V_{in}(\omega)} = \frac{V_{out}(\omega)}{I_{in}(\omega) \times Z_{FB}} = \frac{I_{out}(\omega)/g_{m1}}{g_{m2} \times I_{out}(\omega) \times Z(\omega) \times Z_{FB}} = \frac{1}{Z(\omega)} \times \frac{1}{g_{m2} \times g_{m1}} \times \frac{1}{Z_{FB}} = Y(\omega) \quad (3.15)$$

The Z_{FB} represents the impedance of feedback resistor and has a pre-set value, g_{m1} and g_{m2} represent the gain or sensitivity of each V2CC. Thus the added AFE circuit has introduced a critical change in the impedance estimation process which is implemented by AD5933. Additionally, a number of V2CC output currents stability were tested through a few different types of V-I converters and the Improved Howland Current Pump shows the best stability. In order to achieve the bioimpedance measurements without detectable electric influence on the cell during cell culture process the AFE must block the flow of DC current through the CUS while simultaneously ensuring that the value of the AC current complies with the safety regulations imposed by IEC-60601. All these must be provided by the AFE while keeping the signal input and output signals of the AD5933 circuit within operational levels, i.e. avoiding current or voltage saturation.

3.2.5 Switching Matrix Array Unit

From a multi-electrode based data collection system perspective, by having four electrodes or other multi-electrode setup in each culture chamber and electrodes with different functionality with respect to experimental needs, the system is unnecessarily complicated as is shown in Figure 3.20. It is therefore a number of existing switching units were assessed, including conventional multiplexer and 8×16 digital switching array (Zarlink MT8816EA, USA).

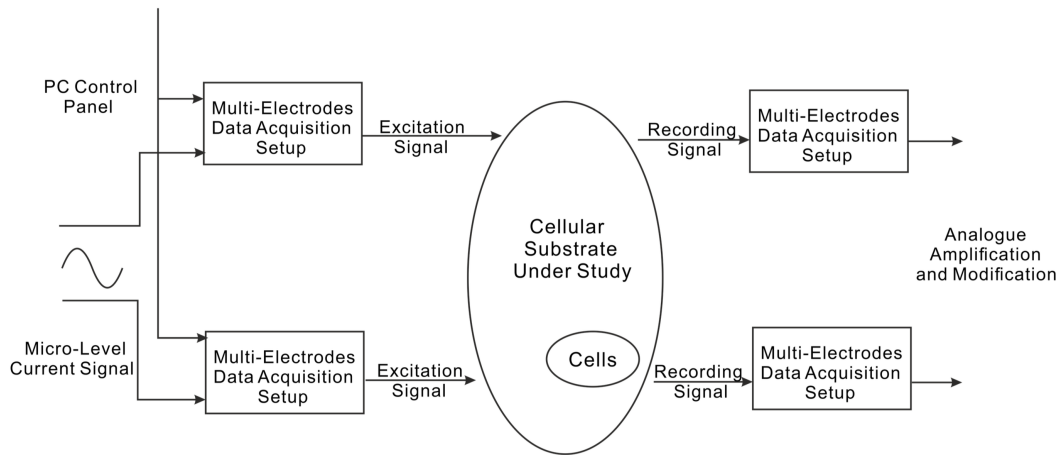


FIGURE 3.20: Proposed Tetrapolar Data Acquisition system with Analogue Front End Circuitry

3.2.6 System Integration and Distance Effect Investigations

The automated system was integrated after full investigation of electrode characterisation and distance effect. A GUI was also designed in Visual Basic platform with capability to provide a set of different valued output excitation signal and equivalent circuit model. An external clock was added to the system to regulate the sequence of exciting and measuring electrodes with pre-set time interval. Once the system was fully fabricated, the distance effect between two potential electrodes was evaluated. The optimal position for electrodes on microbio reactor platform was assessed by utilising a 3D printer [ORDbot Quantum, USA]. The precise movements of measuring electrode in culture chamber were commanded by the 3D printer via a pre-embedded G-Code and the interval for each single shift was setup at exactly 0.2 mm. The bioimpedance was measured from 1 kHz till 1 MHz with 200 Hz interval and incremented for 5000 times at each single position along the movement and the obtained data was compared with the corresponding Signal-to-Noise ratio. The bioimpedance geometrical expression was also utilised to ensure that the value of the AC current complies with the safety regulations imposed by IEC-60601.

3.2.7 DLD-1 Static Cell Maintenance

The cell line used in the tests is DLD-1 which is a human colon carcinoma of epithelial morphology and adherent growth mode. The culture medium used is DMEM High Glucose (4.5 g/l) with 10 % fetal bovine serum and 0.1 % antibiotics. The required seeding density is 10×10^6 cells/ml. Preparation of cells includes resuscitation of frozen cells, passaging cells into a flask and transferring the attached cells from the flask to the PDMS-based microbioreactor. Protocols for these procedures are strictly followed as set out by Public Health England (2013) and sterility is required, so all cell preparation is carried out under a sterile hood using sterilised equipment.

The DLD-1 cancer cells were firstly resuscitated from a -80 °C freezer and were rapidly thawed at 37 °C in a uniformly heated water bath. The cryopreservation medium was then removed by placing the suspension of cells into a centrifuge. The cells were then re-suspended in a warmed culture medium where the culture medium volume was then adjusted to reach the cell seeding density required before transferring the suspension into culture flasks. These were left in an incubator until the cells were estimated to reach a confluency of 60-70 % after which they had to be passaged and the cell media was removed through aspiration. The 5 ml of PBS was then added to the flask followed with a swirl and aspirate operation. A further 2 ml of Trypsin was added to the solution to cover the monolayer of cells and the mixed solution was then incubated at 37 °C for 15 minutes which caused cells to be dislodged. An 8 ml of cell media was then added to neutralise the trypsin reaction and the cells were then transferred to a sterile centrifuge tube for separation. Afterwards, the cells were counted using a haemocytometer with Trypan Blue dye as described above, a microscopic image was taken before transferring them into the PDMS-based microbioreactor to be left at room temperature under the sterile hood for 24 hours.

3.2.8 TrypanBlue Bioassay for DLD-1 Cell

Two patches of microbioreactors with 9 culture chambers were prepared and DLD-1 cells were seeded to each chamber with exactly same 10×10^6 cells/ml density. The viability test was then carried out every 3 hours through the 24 hour experiment before cell number doubled itself in culture chamber. For each time, a 0.1 ml of cell suspension was aspirated from an uninspected microbioreactor chamber and was added to a mixed solution, where the 0.01 ml Trypan Blue Solution and 0.3 ml of PBS were already combined, and incubated for 5 minutes. It was then extracted 20 μ l of mixed solution and placed it on a haemocytometer under a fluorescent microscope where cells were counted in all four outer quadrants of the haemocytometer grid. The number of unstained cells on the haemocytometer per quadrant were determined based on microscope observations, since the dead cells took up the Trypan Blue stain, and the number of cells per ml and total number of cells were calculated for every three-hour operation. The percentages of viable cells were then determined by using the number of unstained cells divided by the total number of cells multiplied by 100. Simultaneously, a bioimpedance measurement of DLD-1 Cancer cell substrate was also implemented. These short term experiments have been repeated three times at room temperature under the sterile hood.

3.2.9 Data Analysis

Differentiation and determination of bioimpedance and corresponding reactance and resistance is possible by measuring the phase angle. A pure cell membrane mass would have a phase angle of 90 degrees whereas a pure electrolyte solution would have a phase angle of 0 degrees. The phase angle was digitally analysed through Discrete Fourier Transform where the period signal waveform, in time domain, received at the collection point of system can be mathematically represented by sum of a fundamental sinusoids

and a series of harmonics which is known as the Fourier Series:

$$f(t) = \frac{a_0}{2} + \sum_{i=1}^{\infty} (a_n \cos(n\omega_1 t) + b_n \sin(n\omega_1 t)) \quad (3.16)$$

Where a_n and b_n are the amplitudes of each harmonic component n , $a_0/2$ is the dc component, and ω_1 represent the angular frequency defined by the receiving signal period. The frequency spectrum of a periodic function of time $f(t)$ is therefore a line spectrum. The Fourier series stated in Eqn 3.16 can be utilized to mimic the original signal in time domain. The phase angle of bioimpedance at a given frequency is essential and it is often considered as the angle of a signal portion. It is specified in angular degrees and provides a reference to the reference value of the entire signal and can be expressed as Eqn 3.17.

$$S(t) = A \sin(\omega_0 t - \phi); \quad \phi = \int \omega(t) dt \quad (3.17)$$

$S(t)$ is the signal as a function of time, ϕ is the total phase shift in real-time domain and $\omega(t)$ is the frequency shift that may vary as a function of time and dt is a period of time.

$$F(\omega) = \int_{-\infty}^{\infty} f(t) e^{-j\omega t} dt; \quad f(t) = \frac{1}{2\pi} \int_{-\infty}^{\infty} F(\omega) e^{j\omega t} d\omega \quad (3.18)$$

This statistical analysis method is known as Discrete Fourier Transformation (DFT). Furthermore, based on Cole equivalent circuit model discussed in the earlier section (Eqn 3.13), a trajectory shaped representation of Cole-equation is used to analyse the culture status of cellular substrate.

Figure 3.21 shows the shape of the trajectory that the real and imaginary parts of the bioimpedance would follow as the excitation frequency varies. As can be seen, R and $-X$, represent equivalent resistance and equivalent capacitances respectively, whereas the centre of the arc is located either on or below the real axis. The distance between the real axis and centre of arc can be represented by the angle, ϕ , which was determined by DFT.

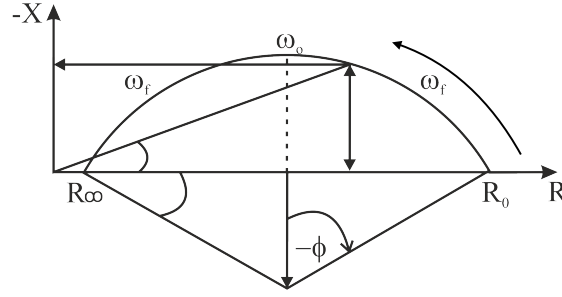


FIGURE 3.21: The trajectory of the real and imaginary parts of bio-impedance varies with the excitation frequency

3.3 Results and Discussions

3.3.1 Electrode Characterisations via Cyclic Voltammetry

As it was discussed in Section 3.1.2, the measured admittance Y contains both the polarization of impedance of working electrode, Z_{ep} , and a cellular substrate volume zone proximal to the working electrode, Z_{CUS} , which is delimited by the position of reference electrode. The measured proximal or constructional cellular substrate zone corresponds to the equipotential surface shown as a stippled line under the reference electrode. However by employing monopolar data acquisition system, the bioimpedance of the cellular substrate, Z_{CUS} , has been mathematically eliminated in the circuitry transfer function and its magnitude is practically negligible in comparison to the impedance of electrode interface based on the aforementioned Stern theory. The recorded value in 3E setup can be mathematically written as

$$\left(1 - \frac{Z_{ep}}{Z_{FB}}\right) \cdot V_e = V_{out} \quad (3.19)$$

The Z_{FB} is a feedback resistor embedded into system with determined value and the derivative of this equation can be found in Appendix B. The dc current is monitored at each dc voltage level and this methodology has been broadly used in electro-chemistry field and known as Cyclic Voltammetry analysis. Within this section, four electrode

materials, namely silver, gold, platinum and graphite, will be studied under three different cellular culture medium solutions: (1) fresh cellular culture medium without cells; (2) fresh cellular culture medium with suspended DLD-1 cancer cells; and (3) 3-day fully metabolised cellular culture medium with stabilised monolayer of DLD-1 cancer cells. The optimised choice of electrode material will be discussed based on the three-electrode monopolar data acquisition system and used throughout all bio-impedance measurements in this work.

3.3.1.1 CV Analysis for Fresh Medium without Cells

Fresh culture medium without cells is the most simplified aqueous condition under this study and acts as a reference control to other medium solutions. Firstly, a silver electrode was used in the cellular culture medium as it is a relatively cheap material and previously reported to be viable in bioimpedance investigations [Monk 2001]. The voltammetric

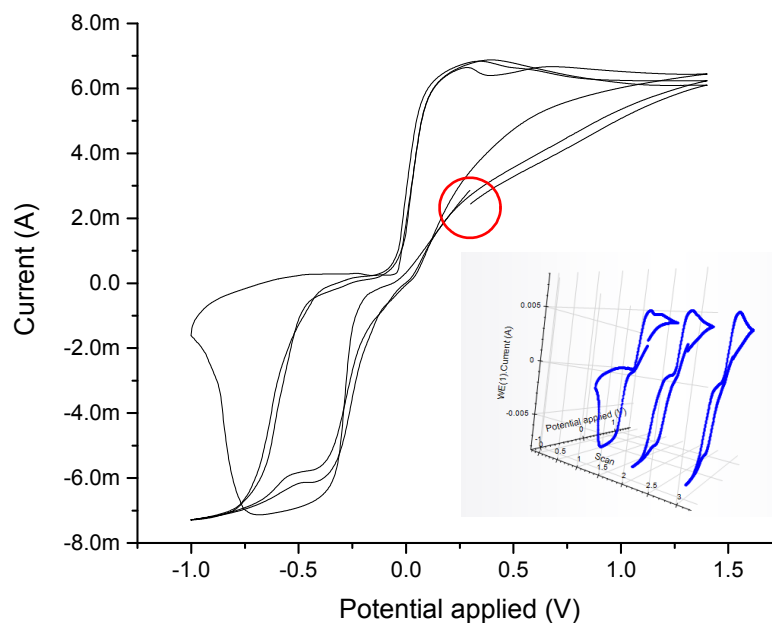


FIGURE 3.22: Three scans of Voltammetric response for a silver electrode in fresh cellular medium. Inset shows the 3D plot of each scan

responses of silver electrode were obtained in fresh cellular culture medium. A 3D plot

can be seen from Figure 3.22, where each curve illustrates the cyclic voltammogram for a silver electrode in 385 μl medium solution at scan rate of 0.1 V/s. The primary horizontal axis indicates the number of cyclic scans. The secondary horizontal axis reflects the defined DC potential applied to the silver electrode in the culture medium recorded as a function of time, and the vertical axis represents the measurement of current at the electrode interface at each excitation voltage level correspondingly in the culture medium. It can be seen explicitly that throughout the first to third scans there are significant reductive and oxidative currents occurring on the silver electrode, and such processes persist after three scans. An alternative 2D image for silver electrode data is also shown in Figure 3.22 to qualitatively identifying the redox current generated at the interface on the electrode. The steady-state current for both reductive and oxidative step shows a current density of ca. $(44.6 \pm 1.32) \text{ mA/cm}^2$, which is massive, compared with other electrode materials (see later). It has been widely reported that silver metal is a reactive material under applied DC voltage in aqueous solutions [Bard 2000]. The oxidation potential of Ag/Ag^+ is 0.8 V (vs. standard hydrogen reference electrode) and silver ion can form various precipitates with solution anions, eg. chlorides, nitrates, halides etc. Furthermore, the enclosed areas over and under the x-axis are not equal, which indicates that there is a net charge supplied to the system. Consequently, it is not unreasonable to suggest that silver metal is taking part in the redox processes with the fresh cellular medium and gets etched away along with increasing number of cycles. Furthermore, the disconnection of the start and end point for cyclic scans in the plotted diagram also indirectly indicate the surface of silver electrode has been irreversibly modified after successive exposure to applied DC voltages. To confirm this point, the silver wire has demonstrated the loss of metal shine after only a single scan under culture medium solutions.

Therefore, despite the previously reported usage of silver electrode in the literature the results shown here have suggested silver is not bio-compatible and cannot be exposed in

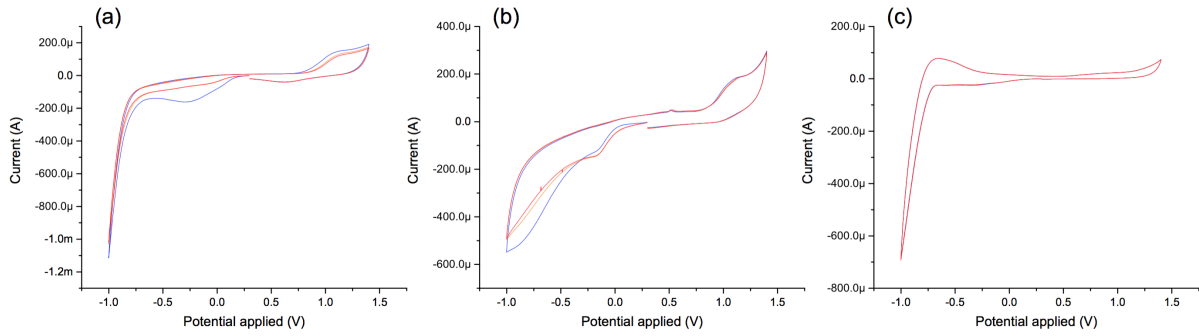


FIGURE 3.23: 2D data plot for a) gold; b) graphite; c) platinum electrodes voltammetric response in fresh medium only over multiple scans. In each diagram Blue: 1st cyclic scan; Orange: 2nd cyclic scan; Red: 3rd cyclic scan

cellular culture medium.

Next, gold, graphite and platinum electrodes are further examined in fresh culture medium without cells. The cyclic voltammetry responses of gold, graphite and platinum electrodes are displayed in Figure 3.23 (a) (b) (c) respectively. The voltammetric responses of these three electrodes are in comparison to that of the silver electrode, where the former shows a smooth connection of both starting and end points after repetitive scans and the latter demonstrates a large disparity due to the significant change of electrode surface. Furthermore, the current density of these three electrodes ranges from ca. 18.4 to 84.5 $\mu A/cm^2$, which is significantly smaller than that of the silver electrode. Consequently, it suggests that voltage excitation at this defined potential window only results in a minimum change of electrode interface on gold, graphite and platinum surfaces.

Both gold and graphite electrodes have shown noticeable reductive and oxidative peaks at ca. -0.2 V and +1.0 V, and larger incremental resistance R , where R is the tangent to the curve at a given point, among those three comparative electrode material in the negative voltage excitation region. These processes are likely to result in a change of medium composition after repetitive measurements and lead to a change of electrode interfacial surface at the applied DC voltage window. In contrast, platinum electrode demonstrates a clean response at the exact same potential window. The absence of

redox processes here is likely related to a combination of competing processes of both the molecule adsorption from the culture medium and oxygen molecule dissociative adsorption on the platinum surface [Sawyer 1982]. Moreover, a sharp increase in current beyond -0.7 V on both platinum and gold electrodes is due to solvent breakdown and formation of hydrogen gas at extreme negative voltage. Furthermore, Figure 3.23 (c) reflects the platinum surface stability upon repetitive scans as the three scans are identically overlapped to each other.

3.3.1.2 CV Analysis for Fresh Medium with Cells

A further set of biological experiments were implemented for gold, graphite and platinum electrodes characterisation with $385\text{ }\mu\text{l}$ of fresh culture medium with suspended DLD-1 cancer cell (10^7 cells/ml density) in PDMS.

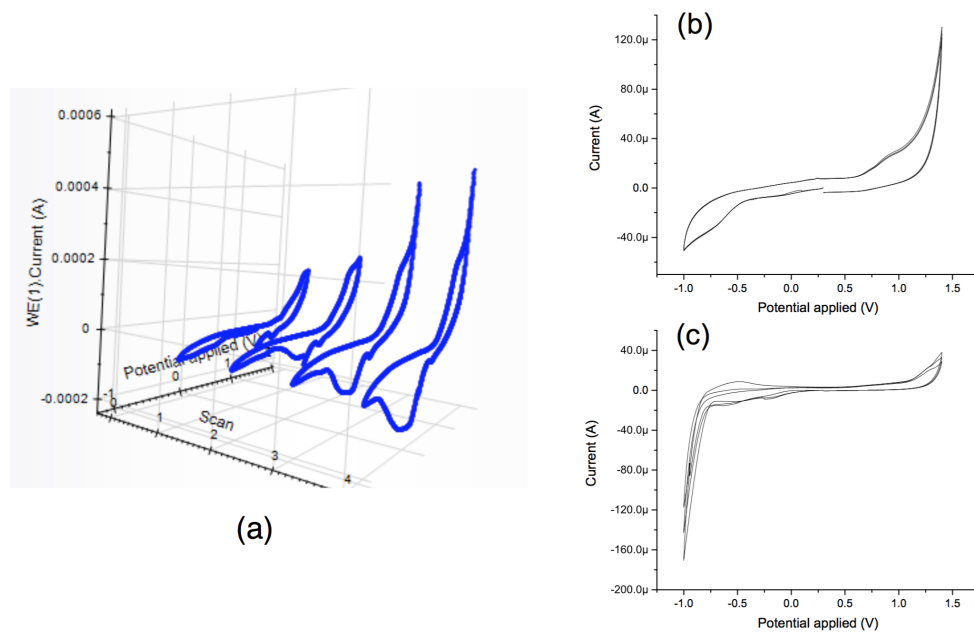


FIGURE 3.24: Voltammetric response in fresh medium with unsuspended DLD-1 cell over continuous multi-scans a) 3D data plot for gold electrode; b) 2D data plot for graphite electrode; c) 2D data plot for platinum electrode

Although the gold electrodes reflected a much smaller incremental resistance in fresh culture medium without cells (Figure 3.23 (a)), Figure 3.24 (a) shows the gold surface

has certainly formed a new layer on the electrode surface with extracellular solution of unsuspended DLD-1 cells as its impedance values change in each cyclic scan. Therefore, it is concluded that gold is not an ideal electrode for biological substrate detection under these circumstances.

Figure 3.24 (b) and (c) demonstrate that after the second scan the cyclic responses start to overlap. Such a phenomenon reflects a good consistency and durability in terms of interface modification on both graphite and platinum electrodes over multiple scans within cellular substrate. It is likely due to a mixture of surface oxidation, adsorption and organic redox processes that reduce the polarisability of both graphite and platinum electrodes and increase the applicability and sustainability of using such electrode materials in biological detection in cellular substrate. Although the interfacial effect can be reduced by employing tetrapolar data acquisition system and no current will flow through two potential measuring electrodes. Nevertheless, the interfacial effect inevitably introduces an additional impedance to current electrodes and hence distorting the quality and consistency of injecting current within BIS system.

Moreover, it is of interest to note that the platinum appears to have a much smaller capacitive current and less incremental resistance at the interface with cellular substrate within ± 0.4 V region. It can be advantageous for bioimpedimetric measurement as minimum current interference resulted from electrode interfacial region will be introduced.

3.3.1.3 CV Analysis for Metabolic Medium with Cells

A voltammetric analysis was implemented on graphite and platinum electrodes within a 3 days fully metabolised static cell culture medium with DLD-1 cancer cell in PDMS microbio reactor. The medium remained unchanged during this culture period. Cultured DLD-1 cells were observed via microscope and confirmed a fully attached thin layer

formed on a 0.25 mm matri-gel scaffold. The formation of the cellular monolayer at the bottom of culture chamber was also observed with doubled cell number over a 5-day culture period. The results in Figure 3.25, shows that the platinum electrode surface reflects a better consistency over multiple scans with old medium and cellular substrate whereas the graphite electrodes tends to be less reactive on the positive potential range between 1.1 V and 1.4 V as each cyclic scan took places. It was noticed that within the voltage range from -0.4 V to +0.4 V, both platinum and graphite electrodes contain small enough capacitive current, ranged within 2.5×10^{-7} to 2.5×10^{-4} ampere (convert to current density by dividing area consistent with previous argument) and both type of electrodes have very small incremental resistance within this voltage range.

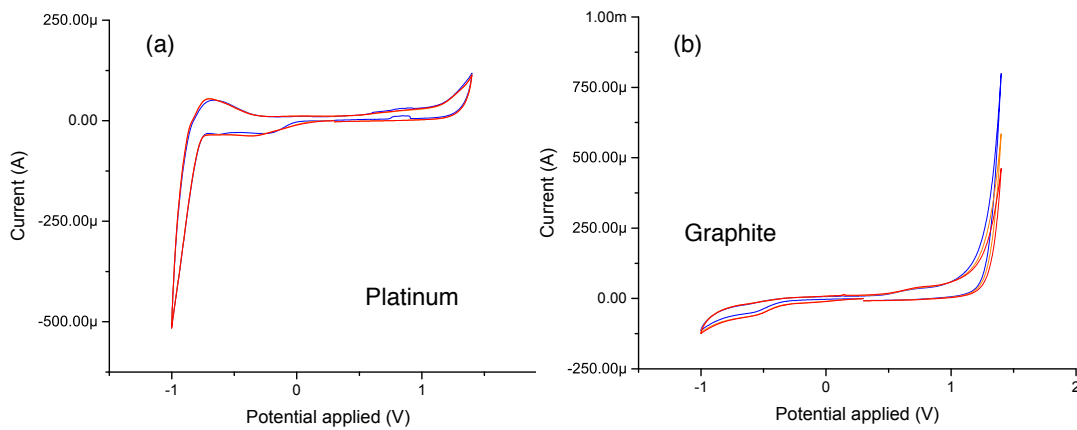


FIGURE 3.25: Voltammetric multi-scan for electrodes in 5 days statically culture DLD-1 cellular substrate with cell density 10^6 in PDMS microbio reactor. a) platinum; b) graphite. Blue: 1st scan cycle, Orange: 2nd scan cycle; Red: 3rd scan cycle

Nevertheless, it is discovered that within -0.4 V to +0.4 V voltage range the platinum electrode has a much reduced incremental resistance and smaller capacitive current under a long term culture circumstances compared with its voltammetric response in fresh medium, shown in Figure 3.26 (a). Conversely, the graphite electrode shows a more reactive redox process and much increased capacitive current under fully metabolised cell culture solution compared with those features in fresh medium. Therefore, overall

the platinum electrode is experimentally proven to be the best electrode choice for long term cell culture monitoring in an oxygen permeable microenvironment.

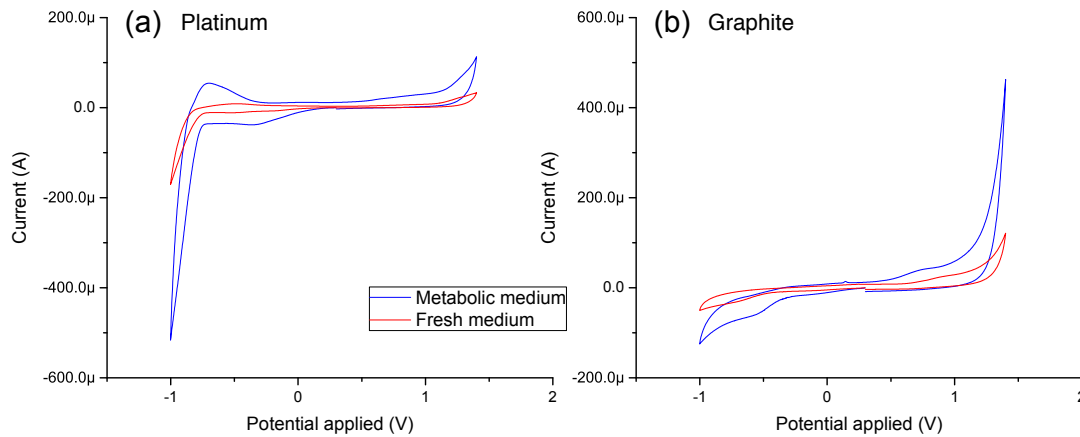


FIGURE 3.26: Voltammetric analysis comparison for electrodes between fresh and old medium. a) blue: platinum in old medium; red: platinum in fresh medium; b) blue: graphite in old medium; red: graphite in fresh medium

It is concluded that by utilising the adequate Stern theory and related model via three-electrode monopolar recording system, the direct electrochemical investigation of electrodes suggests that in a biological cellular substrate solution, the silver electrode is the worst possible choice due to its reactive nature in culture medium aqueous solution. Although the gold electrode demonstrates good voltammetric responses in medium solution without cells, it unexpectedly reflects a similar surface modification issue within suspended DLD-1 cellular substrate in PDMS microbio reactor. Despite the fact that both graphite and platinum electrodes show consistency and durability under cellular conditions within fresh medium, the suitability of the electrode of choice gets differentiated under long term culture circumstances. Chemically speaking, the graphite electrode shows an increase in reactive redox processes and capacitive current within -0.4 V to $+0.4\text{ V}$ voltage range as the metabolic process takes place in cellular substrate whereas platinum performed oppositely under the same environment. Therefore, platinum is experimentally proved as the best electrode choice for long term cell culture monitoring in an oxygen permeable microenvironment.

3.3.2 Analogue Front End Circuitry Validation

In the previous section, electrodes with different material were investigated via cyclic voltammetric response methodology and, the most biocompatible Pt electrode material was identified through experiments. The analysis was further carried out for analogue front end investigations. Pancotto et al. (2016) recently reviewed improvements of AFE for bioimpedance spectroscopy monitoring over last decade includes publications from Valente et al. (2016), Kim et al. (2015) and Yufera et al.(2010). In short most of literatures either chose floating type of ground type of Voltage to Current Converter (V2CC) such Howland Circuit, but the stability of circuitry over large range frequencies and effect of resistance mismatches are generally neglected.

3.3.2.1 First Order Active High Pass Filter Validation

The first order active high pass filter was chosen not only to actively control and stabilise the output signal of AD5933EVAL but more importantly to eliminate the bias DC current introduced by the AD5933 chip. The illustration of circuitry is illustrated in Figure 3.27

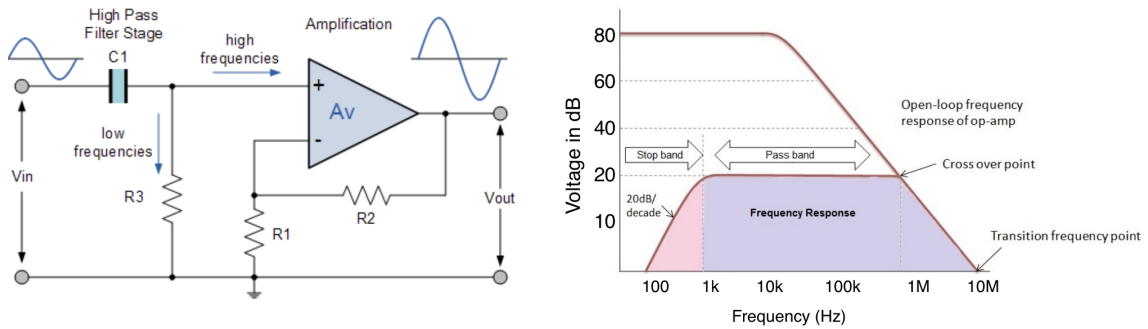


FIGURE 3.27: Illustration of First Order Active High Pass Filter (a) Equivalent circuit; (b) Measured frequency response

The cut-off frequency was set to 750 Hz by defining the R_1 and R_2 to be 100 $k\Omega$ and 1 $k\Omega$ respectively, while R_3 is set to be 2.08 $k\Omega$ and the capacitor has a magnitude of

0.1 μF . The voltage gain under the frequency response of the first order active HPF is given as

$$VoltageGain, (AV) = \frac{V_{out}}{V_{in}} = \frac{A_F(\frac{f}{f_c})}{\sqrt{1 + (\frac{f}{f_c})^2}} = \begin{cases} < A_F & \text{if } f < f_c \\ = A_F/\sqrt{2} & \text{if } f = f_c \\ \cong A_F & \text{if } f > f_c \end{cases} \quad (3.20)$$

Where A_F is the gain of the filter during the defined passband, f is the frequency of output signal of AD5933 that sweep within a selected frequency range and the cut-off frequency, f_c , was previously defined. On the other hand, the circuit gain in steady state in high frequency, A_v , is determined by ratio between $-R_2$ and R_1 and bandpass gain is found to be equal to 1 as $A_F = (1 - A_v) = 1 + R_2/R_1 \cong 1$. The measured frequency response (with chip AD844CN) is illustrated in Figure 3.27 (b) and passband range in frequency domain is range between cut-off frequency and frequency of crossover point between active measured voltage gain and open-loop frequency response of the op amp as it is illustrated in the diagram. It is noted that voltage gain is presented with unit dB and was obtained through the following equation.

$$A_v(dB) = 20(\log_{10}(\frac{V_{out}}{V_{in}})) \quad (3.21)$$

Then the functionalities of HPF were tested as it will only allow signals above 1 kHz to pass through without distortion, thus blocking the possible pathway for any biased DC signal. Moreover, the stability of voltage gain in frequency domain revealed the circuitry successfully stabilised the output signal of AD5933 with unit gain in the bandpass from 1 kHz till 9 MHz .

3.3.2.2 Voltage-to-Current Converter Validation

To evaluate the feasibility of circuitry to act as stabilised current source for portable BIS measurement, a number of circuitry arrangements were realised and verified, including floating load converter, grounded load converter and improved Howland current pump. From a reliability perspective, the stable output current was found by employing two Improved Howland Current pumps with AD844N chip where the structure is shown in Figure 3.28.

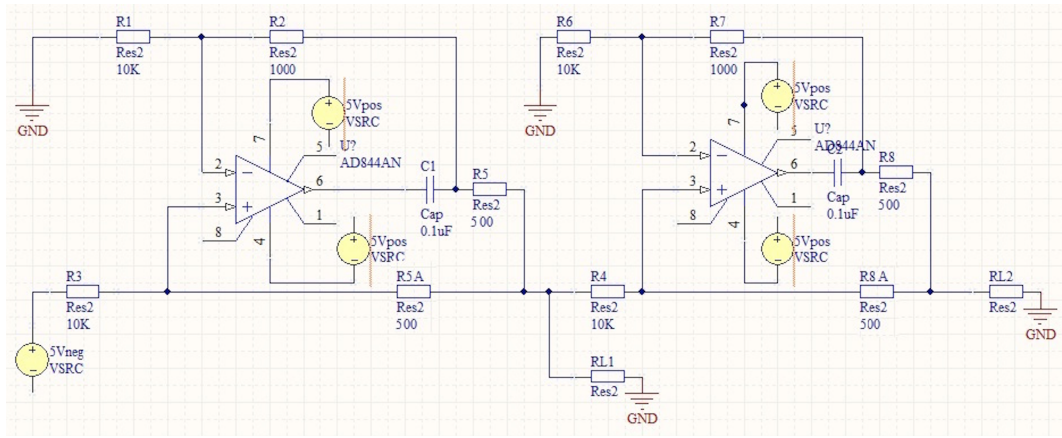


FIGURE 3.28: Illustration of V2CC-based Data Acquisition Analogue System

3.3.2.3 Effect of Resistance Mismatch

A V2CC accepts an input voltage V_{in} and yields an output current of the type $i_o = gmV_{in}$ where gm is the gain in amperes per volt. In short the transfer characteristics of a single improved Howland current pump is expressed as

$$i_o = \left(\frac{R_2/R_1}{R_{2B}} \right) V_{in} \quad (3.22)$$

The sensitivity of the characteristics of the circuit is mainly triggered by R_{2B} , indicating that R_{2B} can be chosen at a relatively small value while the remaining resistances are kept high in order to conserve power. The output current of single improved Howland

circuit is shown in Figure 3.29 where the quality of output current is suggested to be stable not only in the sense that the measured current begin to drop at 1 MHz , which offers a reasonable bandpass, but also in the way that the measured current value is indifferent with the expected current value in the effective frequency region between 750 Hz till 1 MHz .

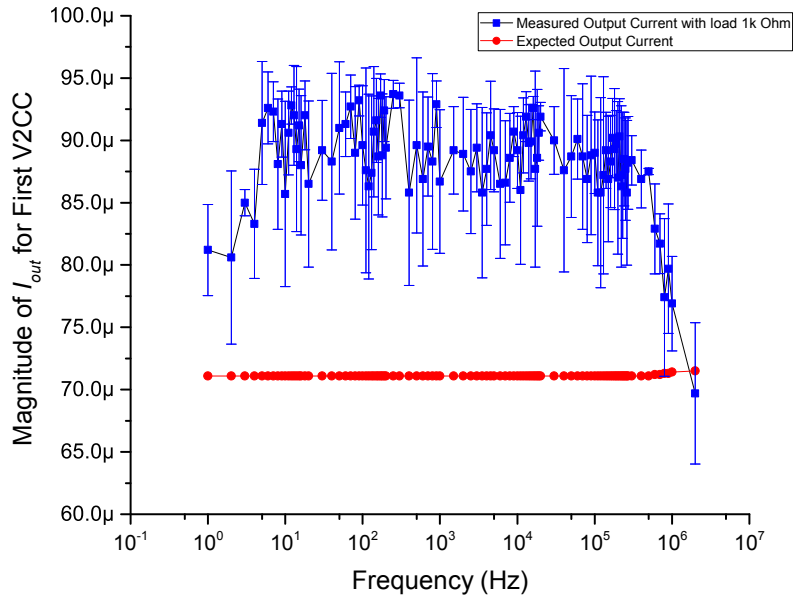


FIGURE 3.29: Demonstration of output current over the frequency from 750 Hz to 100 kHz for 1st V2CC with 1 $k\Omega$ load. Due to the IEC-60601 regulation, the desired current which passes through CUS is strictly controlled under 95.0 μA which fully complies with IEC-60601 for measurements above 1 kHz .

The voltage compliance is approximately $|V_L| \leq |V_{sat}| - R_{2B} |i_o|$ and by considering the Eqn. 3.24, this can be written as $|V_L| \leq |V_{sat}| - R_2/R_1 |v_{IN}|$. For the purpose of extending the compliance of circuitry, R_2 was desirable to be kept sufficiently smaller than R_1 with ratio 1:10. Thus the values of R_1 , R_{2A} and R_{2B} were set to 10 $k\Omega$, 500 Ω and 500 Ω respectively. For practical converter the more realistic formula is often written as

$$i_o = gm \times V_{in} - \frac{1}{R_o} V_L \quad (3.23)$$

Where gm is the gain, or sensitivity, of the circuit, in amperes per volt, V_L is the voltage developed by the output load in response to i_{out} and R_o is the converter's output resistance as seen by the load. For an ideal functioned V2CC, i_o must be independent of V_L and thus R_o must be infinitely large. However, the resistive bridge is likely to be unbalanced because of resistance tolerance. This inevitability degrades R_o which should be infinite for true current-source behaviour. It is therefore of interest to estimate the worst-case value of R_o for a given resistance tolerance specification.

$$\frac{R_4}{R_3} = \frac{R_{2A} + R_{2B}}{R_1}(1 - \epsilon) \quad (3.24)$$

Where the ϵ is the imbalance factor and the R_o in practical case can then be written as $R_o = R_1/\epsilon$. The actual resistors introduced to the Improved Howland Circuit under DC condition were recorded as 10.2 k Ω , 495 Ω , 507 Ω , 10.5 k Ω and 992 Ω respectively and hence the imbalanced factor of circuit in worst-case was found to be 0.029 and the magnitude of output resistance, R_o , is equal to 1.108 M Ω which is large enough in the worst case to eliminate the loading effect in the feedback loop. Furthermore, the common-mode rejection ratio (CMRR) often used to validate the circuit system stability where its expression is shown in Eqn 3.25

$$CMRR_{dB} \simeq 20 \log_{10} \left| \frac{1 + (R_{2A} + R_{2B})/R_1}{\epsilon} \right| \quad (3.25)$$

The CMRR of a practical difference amplifier is actually the result of two effect: bridge imbalance and op amp non-ideality and was calculated to be 36.90 dB which indicates the resistors in circuit is relatively tightly matched. The two effects are interrelated so that it is possible to unbalance the bridge in such a way as to approximately cancel out the effect of the practical op amp.

3.3.2.4 Validation of Loading Effect

Other than CMRR, the dependency of loading resistance for piratical V2CC was also examined. A number resistance tolerance versus frequency was measured by precise LCR meter prior to the output current consistency test.

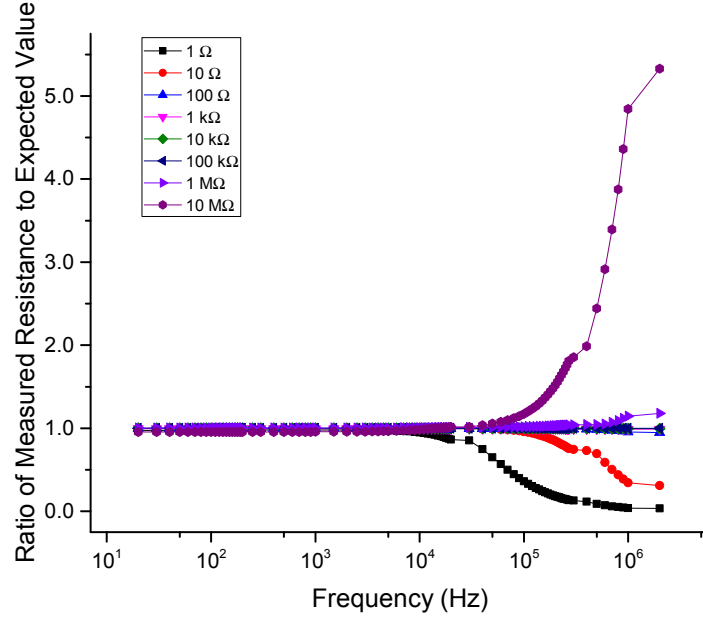


FIGURE 3.30: The ratio between measured resistance of practical resistor and its theoretical values in the frequency range from 20 Hz to 1 MHz

As it can be seen in the Figure 3.30, the 1 Ω and 1 $M\Omega$ resistor begins to vary as the frequency increases, this is because practical resistors include a series inductance and a small parallel capacitance; these specifications are certainly important to be considered in the control loop for BIS measurement in high frequency applications. Thus, by having obtained the (log-based) characteristics of various resistors over the range of frequency from 20 Hz to 1 MHz , each magnitude of load voltage is measured with corresponding log-scaled frequency, and then the output current is obtained simply using equation:

$$i_0 = \frac{V(f)_{log-scale}}{R(f)_{log-scale}} \quad (3.26)$$

A number of practical op-amp, including OP277, 741CN, 708BD and AD844, were examined in the proposed V-I converter circuit and among these op-amps, the AD844 was found to provide a stable current with the widest frequency range and smallest op-amp impedance under 100 kHz . The results of output current, i_o , of proposed V-I converter with AD844 chip is shown as follows:

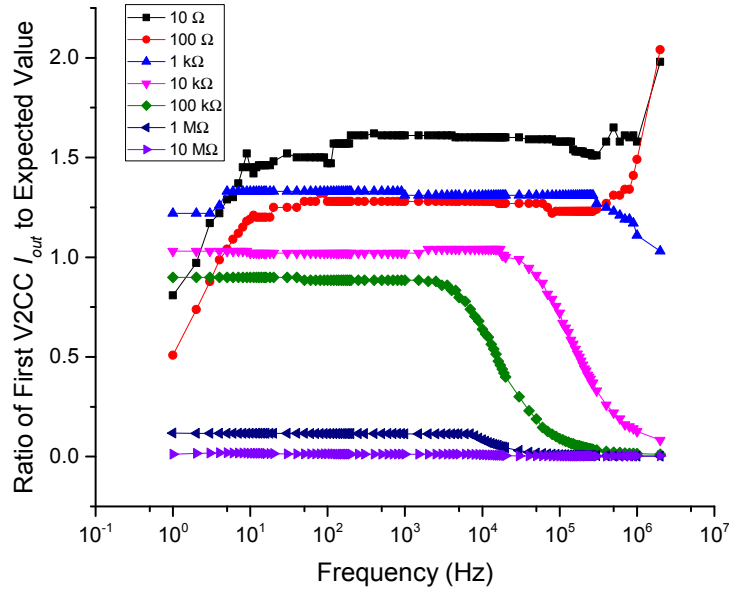


FIGURE 3.31: 1st V2CC output current frequency response performance with different loads

Most of the measured output currents are reasonably close to the expected current, which is set to be $70.7\text{ }\mu\text{A}$. On the other hand, the obtained results also suggested that the V-I converter, which was designed with initial purpose to provide constant current over a wide range of frequency spectrum, reaches its limits as the value of output current is load dependent. However, different magnitude of load impedance value reflects different load dependency characteristics. Both output current with $1\text{ M}\Omega$ and $10\text{ M}\Omega$ load resistor failed to manage to remain at a constant value that is relatively close to expected current. This is because the voltage compliance, as discussed in the previous section, is based on the assumption of symmetric output saturation. It is displayed as $|V_L| \leq (R_1/(R_1+R_2))$

V_{sat} , since in the experiment R_2 is already being set significantly smaller than R_1 , giving $|V_L| \leq V_{sat}$. However, because the system is designed to generate constant current at $70.7 \mu A$ and thus by having very large impedance load, the V_L value can easily reach the saturation limit and thus the system is forced to reduce the output current significantly or even to a negligible extent. The output current with load impedance of $1 k\Omega$ have the best linearity fitting with expected output current till $95 kHz$ whereas output current with $100 k\Omega$ load impedance reflected a slight negative offset with a narrow passband. In contrast, the output current with small load impedance within the range from 10Ω to $100 k\Omega$ all revealed linearity with expected value but with a positive offset. Importantly, the output current of proposed V-I converter with 100Ω and $1 k\Omega$ both showed a good frequency response from $10 Hz$ until $600 kHz$, which is one of the most common impedance ranges for majority types of cellular substrate. Furthermore, both positive and negative output current offset obtained through experiments were later added to the control algorithm corresponding to the estimated cellular substrate impedance.

The reliability of second Improved Howland circuit, which was acting as part of I-V converter, was evaluated by assuming the impedance of inspected sample to be within $1 k\Omega$ range with second load impedance at $10 k\Omega$ and the linearity of measured output current with respect to the expected value is shown in Figure 3.32.

It can be seen that a good linearity was obtained with various load in frequency range until $85 kHz$ where the value is then set as upper limit for effective measurement in the cell culture monitoring experiment. An additional improvement was made by adding two small capacitors with value of $10 pF$ in parallel with R_4 and R_1 . Literature suggests that the Howland circuit employ both positive and negative feedback; they may become oscillatory under certain condition. Adding capacitor in parallel will be adequate to make negative feedback prevail over positive feedback at high frequencies and thus stabilise the circuit.

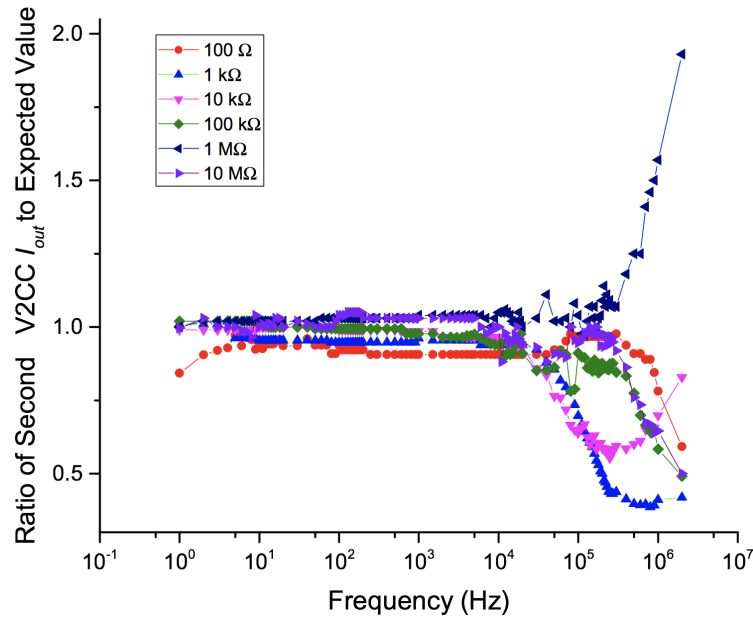


FIGURE 3.32: 2nd V2CC output current frequency response performance with different loads

3.3.3 BIS System Realization

After the device was fully fabricated, a number of system integration and development was carried out before implementing the static cell culture for Bioimpedance measurement. The AD5933 (Analogue Device) impedance analyser, combined with analogue front-end circuit, where its component output currents stability is verified through a few analogue circuit experiments, were used as a replacement for professional bioimpedance measuring instruments. The MT8816EA (Zarlink, US), a digital switch IC collectible DIP 40, was used as the matrix switch to accommodate a large number of different output combinations and enable the integration of future functionalities. An Arduino Uno (C language based) board, with 14 digital input/output pins and a 16 MHz crystal oscillator, was used as the micro-controller for the matrix switch as well as the impedance analyser.

3.3.3.1 Switching Matrix Array

Based on the above ideas and from the perspective of the spaces available for the future development, the decision was made to use the matrix switch array. Therefore, it is essential to utilise the MT8816AE Digital Switch IC collectible DIP-40 as the matrix switch so that a vast number of different output combinations are obtained (for example, MT8816EA can decide where the current is injected into the certain electrode pair while the rest are responsible for measuring potential differences). An Arduino Uno (USB link, C language based) board, with 14 digital input/output pins and a 16 *MHz* crystal oscillator, is used as the micro-controller for the matrix switch. AFE are connected into pin Y_0 - Y_3 on the MT8816EA chip where Y_0 - Y_7 are analogue (input/output), the digital output of Arduino Uno is connected with pins AX_0 - AX_2 and AY_0 - AY_3 where all of these seven pins are digital (input/output) and 16 electrodes are connected with pin X_0 - X_{15} . The digital outputs of the Arduino Uno are connected with MT8816EA pins AX_0 - AX_3 and AY_0 - AY_2 where all of these seven pins are digital (input/output).

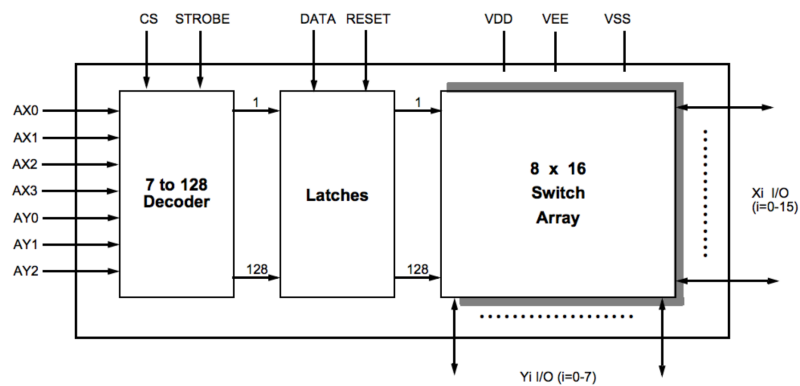


FIGURE 3.33: Functional block for Switching-Matrix-Array

From the Figure 3.33 illustrated that there are 128 output combinations available between Y_i and X_i wherein code Y_i represents the row of switching matrices and X_i represents the column of switching matrices. Thus, by receiving a digital signal from the Arduino Uno board via AX_0 - AY_2 pin it will allow us to use the PC to give a desired

connection order between Yi analogue (I/O) and Xi (I/O) provided it obeys the embedded algorithm. Figure 3.33 address Decode for Switching Array MT8816EA; schematic connections between Arduino Uno and MT8816EA) can be found in Technical Data Sheet.

3.3.3.2 Flow of Control and GUI Development

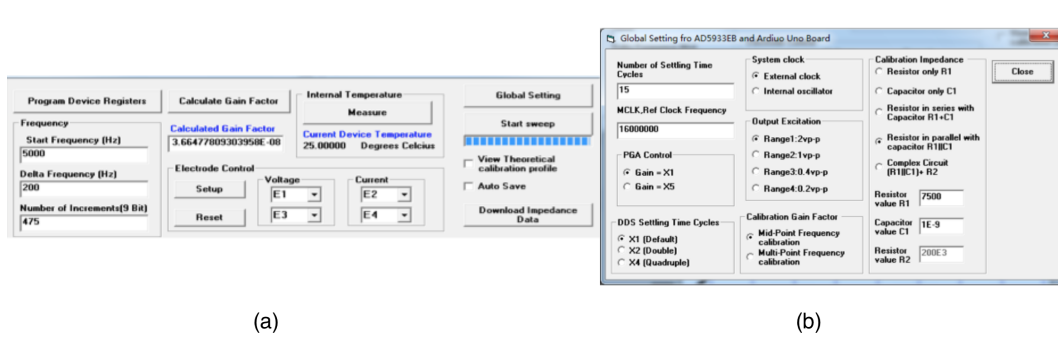


FIGURE 3.34: Designed and developed GUI Interface with capability to select different equivalent circuit model with different Data Acquisition System

The software used to control AD5933 and interface with the PC to calculate and display the impedance measurements and enable data log functionalities were all developed in Visual Basic 6.0. An electrode control function which interfaces with MT8816EA via Arduino was developed which can handle 4 electrodes in operation simultaneously and at maximum could accommodate 16 electrodes in one setup which is shown in Figure 3.34 and the Control Flow of system is illustrated in the Figure 3.35.. An automated clock function was also added to enable multiple frequency sweeping and avoiding clashes caused by propagation delay. A parameter setup process to customise the output value of AD5933 and choose different equivalent circuits for different types of cells were also programmed for future experiments. It is noted that the stop frequency value can be determined by Eqn 3.27.

$$StopFrequency = StartFrequency + (No.ofIncrements \times \Delta Frequency) \quad (3.27)$$

This finalises the expression of actual bioimpedance of CUS and this was taken into account in calculating the coding section:

$$Z_{CUS}(\omega) = \frac{1}{Y(\omega)} = \frac{V_{out}(\omega)}{I_{in}(\omega)} = \frac{V_{out}(\omega)}{V_{in}(\omega)/Z_{FB}} \quad (3.28)$$

$$V_{in}(\omega) = \left(\frac{R_2/R_1}{R_{2B}}\right)^2 \times \left(1 + \frac{R_{HP1}}{R_{HP2}}\right) \times V_{out}(\omega) \times Z_{CUS}(\omega) \times Z_{FB} \quad (3.29)$$

$$GainFactor \cong \frac{V_{out}(\omega)}{V_{in}(\omega)} = \left(\frac{R_{2B}}{R_2/R_1}\right)^2 \times \left(1 + \frac{R_{HP1}}{R_{HP2}}\right)^{-1} \times \frac{1}{Z_{CUS}(\omega)} \times \frac{1}{Z_{FB}} \quad (3.30)$$

Where the R_{HP1} and R_{HP2} are resistors in first order active High Pass Filter, R_1 , R_2 and R_{2B} are resistors setting in the Improved Howland Circuit, Z_{FB} is the feedback resistor embedded in the signal receiving unit, $V_{out}(\omega)$ is the output signal generated from AD5933 with selected frequency range and $V_{in}(\omega)$ is the measured voltage value at input point of AD5933 chip. Thus other than $Z_{CUS}(\omega)$ and $V_{in}(\omega)$, which is measured analogue signal with corresponding excitation frequency, all other parameters include output signal $V_{out}(\omega)$ were all pre-set with desired values and the unknown parameter, $Z_{CUS}(\omega)$, was therefore mathematically identified.

3.3.3.3 Distance Effect Investigation

The precise movements of measuring electrode in culture chamber were commanded by 3D printer via a pre-embedded G-Code and the interval for each single shift was setup at exactly 1 *mm*. The impedance of culture medium was measured in the separate chamber to providing a base value prior to the bioimpedance measurement. The bioimpedance was measured at each single position along the movement and the obtained data was compared with the corresponding Signal-to-Noise ratio and it is shown in Figure 3.36.

A strong linearity is apparent between bioimpedance and the distance between referential electrode and potential measuring electrodes and the Cole-Cole plot is illustrated

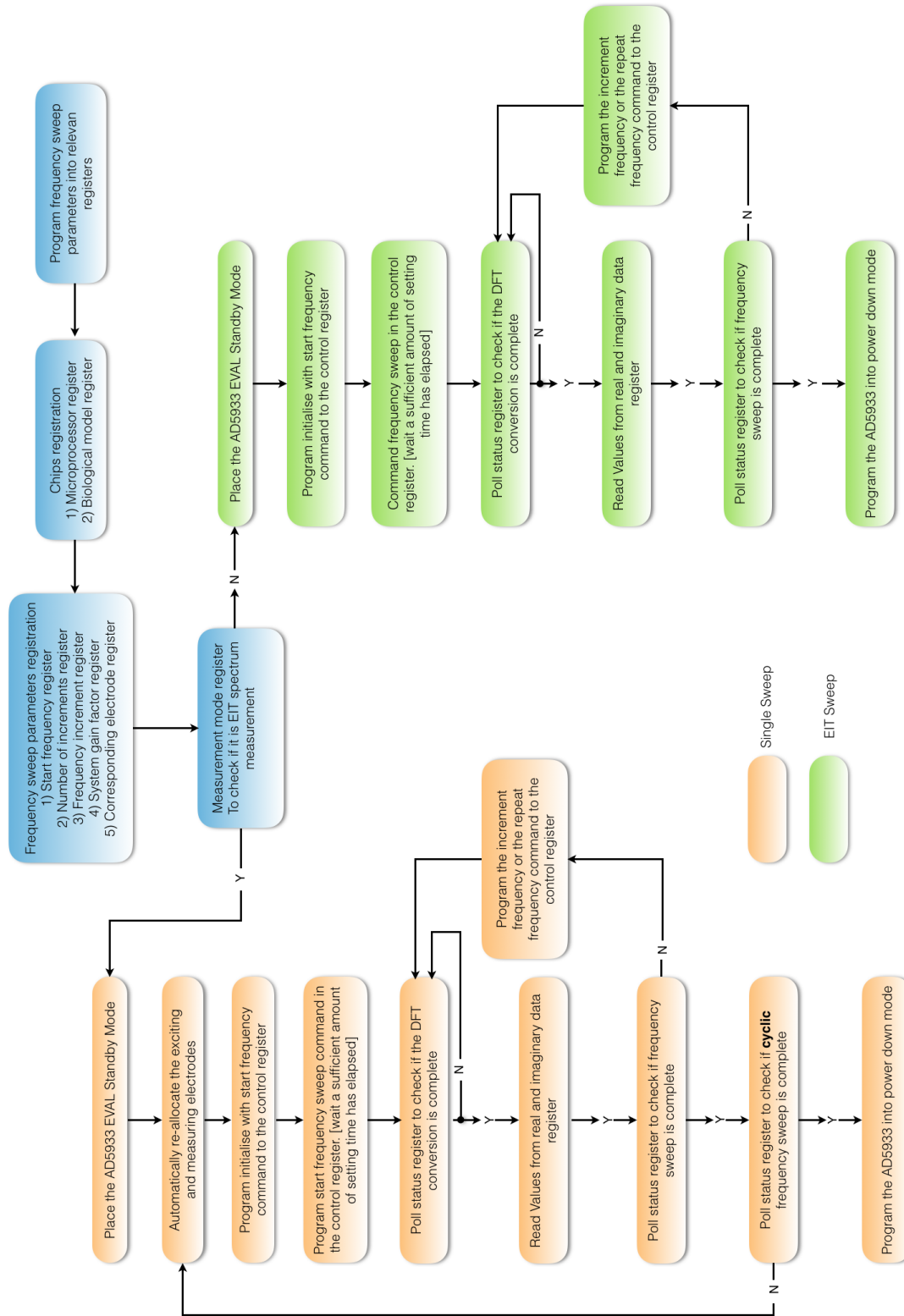


FIGURE 3.35: The Control Flow for Miniaturized BIS measurement

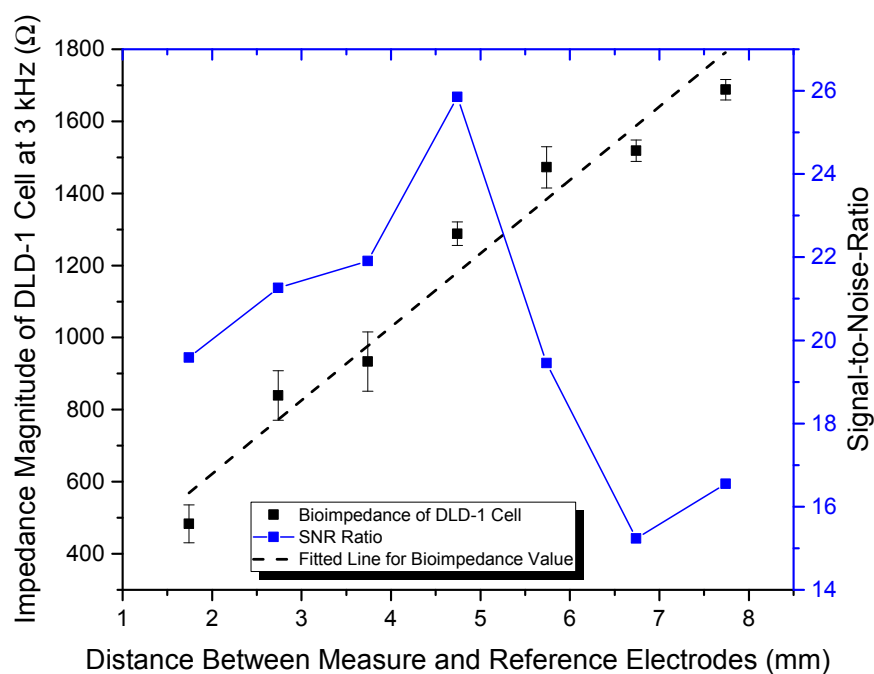


FIGURE 3.36: Magnitude of Bioimpedance measured via Tetrapolar Data Acquisition setup and SNR value versus distance between measuring electrodes and reference electrodes

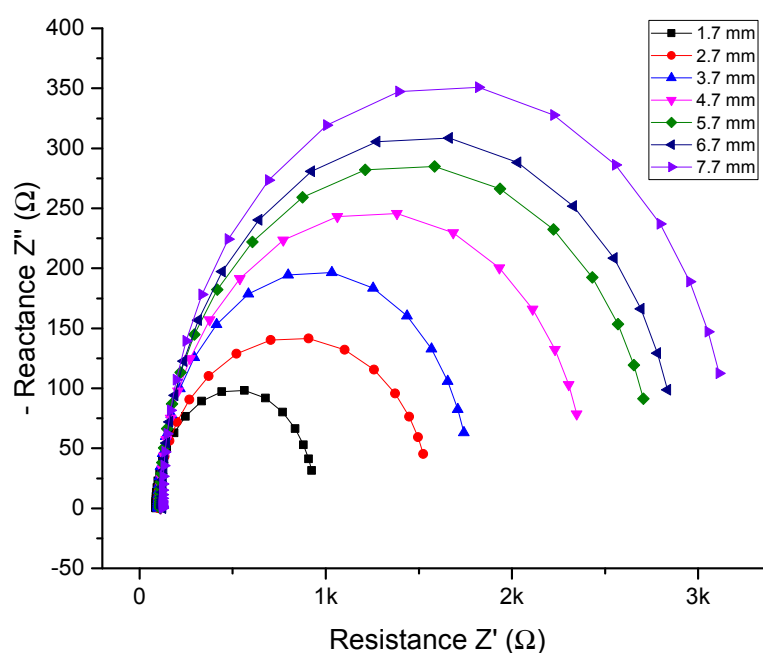


FIGURE 3.37: Bioimpedance spectrum plot with different distance between measuring and referencing electrode in Tetrapolar Data Acquisition setup

in Figure 3.37 where the radius of the plotted curve represent the absolute magnitude of Bioimpedance. This linearity not only agrees with the geometric expression of bioimpedance explained in Section 3.1.5.1 but also proves that the voltage and current relation has a linear characteristic and hence the applied electrical field through existing data acquisition system does not exceed the non-linear frequency threshold. The SNR ratio was obtained through the relevant statistical signal processing in Matlab and it can be clearly stated that with the distance between two potential electrodes greater than 5 *mm*, the SNR drops drastically from 25.8 *dB* to 14.7 *dB*. This is because as the distance increases, despite the monitored bioimpedance increasing accordingly, a more distorted noise signal is likely to be recorded and amplified through the measurement, and hence reduce the SNR ratio. In contrast, when two potential measuring electrodes were too close to each other, the noise signal is reduced during the data recording. Nevertheless the bioimpedance value has also experienced a reduction with high decreasing gradient. As a result, the SNR decreased from 25.8 *dB* to 19.2 *dB*. Thus the optimal distance between two potential electrodes was found to be 5 *mm* while the two current electrodes were set to 12.5 *mm* from each other.

3.3.3.4 Data Acquisition Setup Effect

As it was explained in Section 3.1.8 in this Chapter, in contrast with the two electrode methodology, no current flows through the potential measuring electrodes and the injected current flows completely through the biological cellular sample and hence the detected potential difference across the sample is directly proportional to the electric response of cellular substrate. To evaluate the difference between bipolar and tetrapolar data acquisition system, a number of test were implemented to measure both physical material as well as biological cellular substrate.

With normal *C* and *RC* circuits, both of the results show that, in the 2E measuring system, the impedance of the circuit increases at a high frequency range (i.e. around 12.5

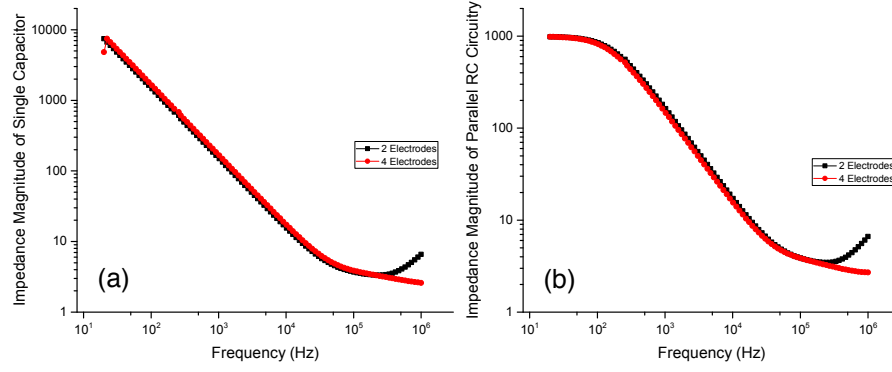


FIGURE 3.38: Electric Impedance Measurement Over Frequency with both Bipolar and Tetrapolar setup (a) Single capacitor; (b) RC Parallel circuit

kHz). This means that, at high frequency range, the circuit also contains inductive characteristics which should not be the case. More importantly, the unexpected inductive characteristics disappeared in the 4E measuring system. The similar effect was observed by evaluation experiment with biological cellular substrate. The results demonstrated that in the mid-high frequency range, there is a decrease in bioimpedance, in frequency domain, measured by four-electrode setup compared with those data collected by 2E system as illustrated in Figure 3.39.

Similar effect was also observed for biological substrate impedance measurement with previously identified optimal electrodes position and the results is illustrated in Figure 3.40.

This is because that in the 2E setup the measured voltage at input point of AD5933EVAL chip is largely dependent on the interface impedance of electrodes. The equivalent circuit of data acquisition with 2E setup is briefly illustrated in Figure 3.39 (a) and the current flow through the monitored biological sample is defined as

$$i_o = \frac{V_L - V_{DD}}{2Z_{ep} + Z_{CUS}} \quad (3.31)$$

and the voltage measured at the input point of AD5933 chip can be mathematically expressed as $V_{final}^{2E} = -V_{FB} = -i_o \times R_{FB}$, and the measured voltage is therefore written

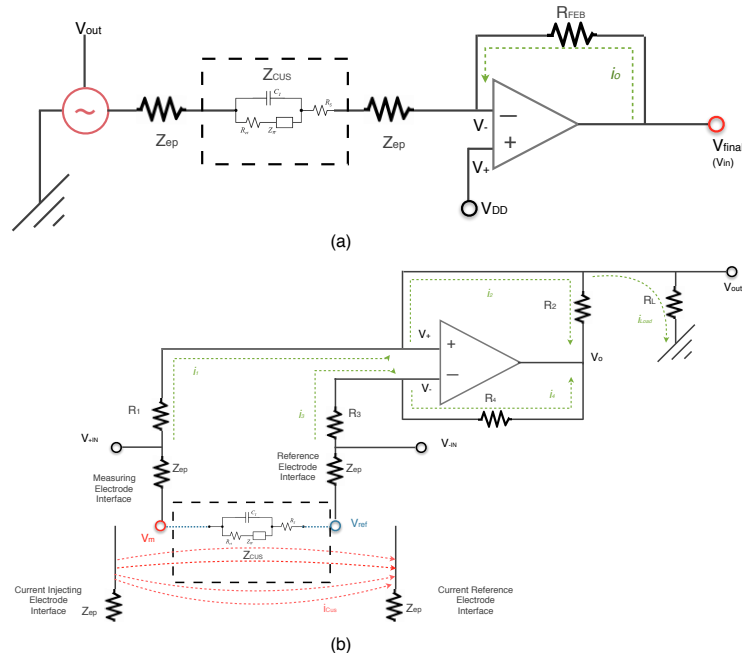


FIGURE 3.39: Equivalent Circuit for Bipolar and Tetrapolar Setup (a) Bipolar; (b) Tetrapolar

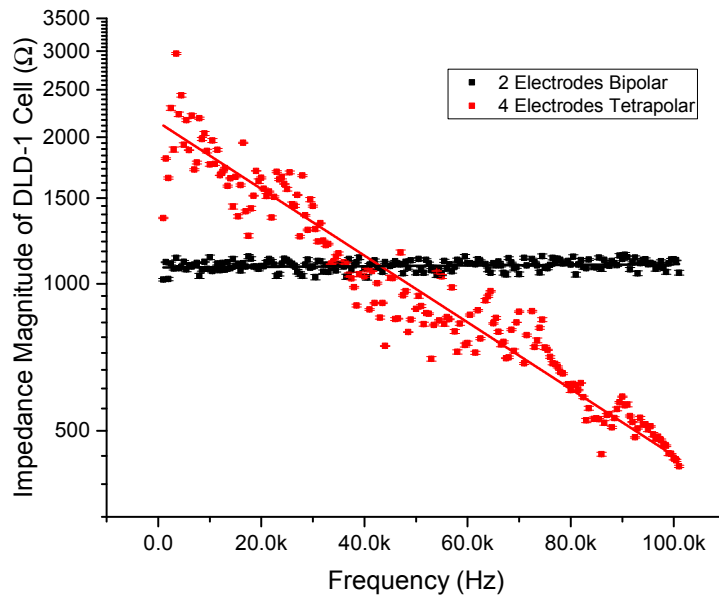


FIGURE 3.40: Biological Impedance Measurement for DLD-1 Over Frequency with both Bipolar and Tetrapolar setup with Optimal Distance between Measuring and Reference Electrodes

as

$$V_{final}^{2E} = R_{FB} \times \frac{V_{DD} - V_L}{2Z_{ep} + Z_{CUS}} \quad (3.32)$$

The R_{FB} , V_{DD} and V_L in the above equation are pre-set constant values, and hence V_{final} is largely effected by Zep since most of Z_{CUS} are small and under one thousand Ohms. In contrast, the recorded value in 4E setup can be mathematically written as

$$V_{final}^{4E} = I_{load} \times R_{Load} = \frac{(V_{+IN} - Z_{ep} \times i_{CUS}) - (V_{-IN} - Z_{ep} \times i_{CUS})}{R_1} \times R_L = \frac{Z_{CUS} \times i_{CUS}}{R_1} \times R_L \quad (3.33)$$

All parameters except bioimpedance of cellular substrate, Z_{CUS} , were either set or strictly controlled as constant values and thus the measured voltage at input point of AD5933 chip is only dependent on the magnitude and phase shift of Z_{CUS} . The deviation of above equation is discussed in Appendix C. Three static cell culture tests were carried out using the DLD-1 cell line culture to test the short term operation of the system and whether the bioimpedance measurement system is suitable for cell culture viability determination.

3.3.4 Static Cell Culture for DLD-1 in Microbioreactor and Viability Assessment

The DLD-1 cells were then re-suspended in a warmed culture medium where the culture medium volume was then adjusted to reach the cell seeding density required before transferring the suspension into culture flasks. These were left in an incubator until the cells were estimated to reach a confluency of 60-70 % where the contact inhibition between cells were taken place. The attached DLD-1 cells were transferred into PDMS-based culture chamber array (9 chambers) from the flask with seeding cell density of 10×10^6 cells/ml for each chamber and was left at room temperature under the sterile condition for 24 hours. The viability tests were carried out every 3 hours through 24

hours of PDMS-based cell culture experiment. For each time, the cells were counted in all four outer quadrants of the haemocytometer grid with Trypan Blue solution under a fluorescent microscope to determine the number of cells per millilitre as well as the total number of cells. The averaged percentages of viable cells at different stages for repeated three experiments were then obtained through the number of unstained cells divided by the total number of cells and the results are shown in Figure 3.41.

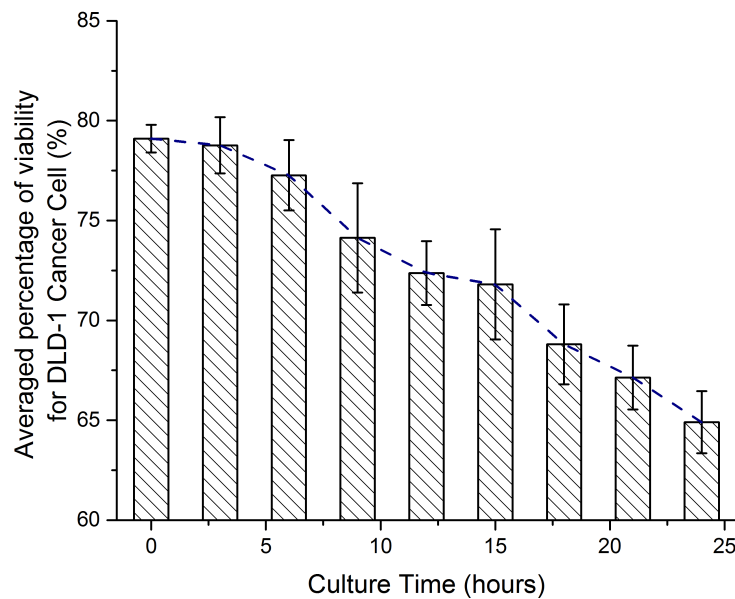


FIGURE 3.41: Evaluation of the DLD-1 cancer cell viability after reaching confluency during static cell culture experiments. The cell viability was evaluated by using haemocytometer with Trypan Blue solution. Results are given as mean $\pm$ standard deviation, $n > 3$ separate experiments. $p = 0.012$ (< 0.05)

The drops of temperature over 24 hours and contact inhibition between cells causes cells to lose their spread shape and become round and caused cells not being able to form a focal adhesion bonds with the bottom surface of PDMS chamber and thus decreases its viability over time. Moreover, the viability test for cultured DLD-1 cancer cell was implemented on a three-hour basis where the aspiration operation is always required. These frequent manual interventions not only break the sterile environment but also bring contamination risks to the culturing cells. The DLD-1 cancer cell was used in the

experiment had been passaged too many times (38 times), where the quality of the cell generally decreases with passaging. The excited current with high frequency occasionally passed through the cell substrate every time when the spectroscopy test is operated and this high frequency may also bring the electric influence to the cellular behaviour during the culture. Furthermore, in the static culture experiment, the consumption of nutrients and the production of metabolites are continuous which causes the composition of culture medium around the cells to vary with time and thus favours formation of a fluctuated and unsteady culture environment that indirectly causes cell death.

The figure depicts a gradually decreasing tendency for the percentage of viability for the DLD-1 cancer cell from 79% to 65% over the repeated 1440 mins static culturing process. A few fluctuations also appear in the decreasing tendency and this could well be because of the uncertainty of the experimental procedure, such as inaccurate aspiration operations, non-uniform distribution of cultured cells as well as uncertainty in the cell count. Nevertheless, the results suggest the existence and significantly increase of the dead cells during the culture process ($p < 0.05$). The obtained data was then correlated with the data from the bioimpedance measurements for validating the proposed viability testing approach.

3.3.5 Feasibility of the Chip-based Bioimpedance Measurement for Cell Substrate

The current driven bioimpedance spectroscopy measurement of DLD-1 cancer cell substrate was implemented on three-hour basis in the microbioreactor chamber. The recorded bioimpedance of the cells substrate remains almost stable, with only a 2% increase in magnitude, as the excitation currents frequency increases. The resistance and reactance were extracted from collected data and calculated based on the bioimpedance measurement within AD5933 chip. The relative permittivity of DLD-1 was also obtained

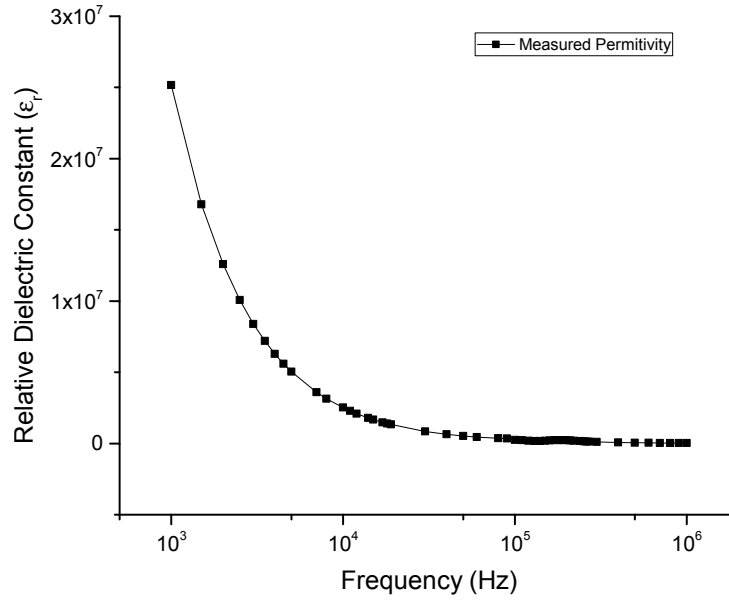


FIGURE 3.42: Identified Relative Permittivity of DLD-1 with Cell Density of 1×10^6 cells/ml

from calculated the equivalent capacitance by employing the geometric model described in Section 3.1.4.2 and is illustrated in Figure 3.42. The tendency of both resistance and reactance agree with the theory which indicates the functionality of the proposed portable bioimpedance measurement system.

Furthermore Cole-Cole model was also generated based the extracted data where the reactance and resistance of the cell substrate were determined and is illustrated in Figure 3.43. For a given cell substrate with constant viability, the reactance and resistance changed over frequency spectroscopy due to the changes of dielectric properties of cell as the excited frequency increases from 1 kHz to 1 MHz and therefore this figure shows the similarity compared with the theoretical Cole-Cole model plot (Figure 3.21).

As discussed in Section 3.1.5.1, the Eqn. 3.9 establishes that resistance decreases as equivalent conductance increases which is caused by the increase of cell substrate conductivity. Similarly, the reactance increases as the equivalent capacitance decreases due to the fact that permittivity of cell substrate drops as frequency increases. Meanwhile, at

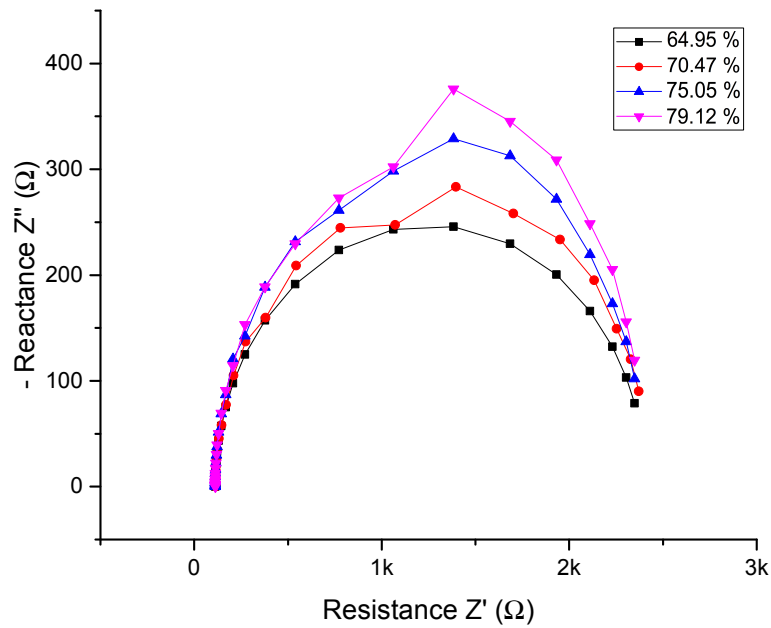


FIGURE 3.43: Cole-Cole plot for DLD-1 Cell with corresponding Viability

a given frequency, the decreases of viability with increases number of death cell which inevitably changes the equivalent circuitry structure of inspected cellular substrate. Thus the Cole-Cole diagram, which contains a number of variables, can only be used to provide general guidance for viability changes within biological cellular substrate. Nevertheless, from the plotted Cole-Cole diagram it suggested the change of biological viability is sensitively reflected for reactance and resistance change particularly within 2 kHz to 20.65 kHz which is considered as effective frequency range for future viability analysis.

3.3.6 Bioimpedance Measurements For the Evaluation of Cell Viability

The impedance spectrum (Figure 3.43) shows a typical electric property of the DLD-1 Cancer cell substrate (density of 10×10^6 cells/ml) and further calculations, including the Cole-Cole plot, were obtained based on the Frick Model, where the cell substrate is re-defined as a combination of a resistor, R_e , that connects in series with a paralleled RC circuit.

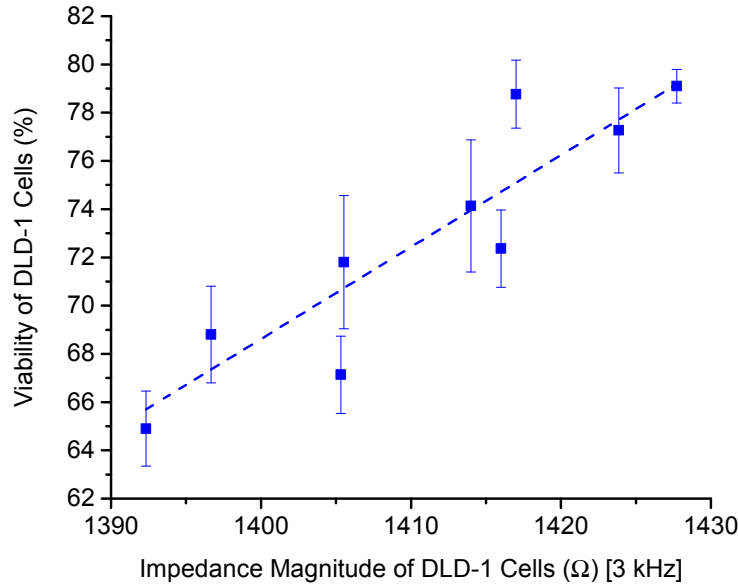


FIGURE 3.44: Correlation between percentage viability of DLD-1 cancer cell and ratio of resistance over capacitive reactance of cell substrate at 3 KHz. ($R^2=0.831$)

The measured magnitude of bioimpedance at 3 kHz for cell substrate decreases from 1.427 kΩ to 1.392 kΩ (2.51 %) with corresponding cell viability in the microbio reactor chamber, where 3 kHz lies within the effective frequency range defined in previous section. The correlation of cell viability and the magnitude of the bioimpedance for a cultured DLD-1 cancer cell substrate was then investigated and is shown in Figure 3.44. From Figure 3.44, the least squares, R^2 , was 0.831 which indicates a reasonably linear relationship between the percentage of DLD-1 cancer cell viability over a 1440 minute culturing process and its bioimpedance magnitude at 3 kHz over the same period of time. A better linear relationship was found between a cells viability and its bioimpedance at high end of β dispersion (250-350 kHz) range, however due to the potential electric effect that a high frequency signal could introduce into the cell substrate, it was suggested to use the excited current within α dispersion ranged frequencies (100 Hz to 10 kHz) for small cell substrate measurement. Furthermore, there are a few values in Figure 3.44 that did not follow the tendency and it was suspected that in the monolayer cell culture system, a high cell density (e.g. 10^7 cell/mL) would cause more cells to be laid on the electrode surface and block the current flow path which reflects a higher bioimpedance.

3.3.7 Cole-Cole Analysis For the Evaluation of Cell Viability

Nevertheless, a significantly better linearity ($R^2=0.957$) was discovered by correlating the percentage viability of DLD-1 cancer cell and the ratio of Resistance over Reactance at 3 kHz which is shown Figure 3.43. Structurally, the cells are membrane bounded compartments filled with a concentrated solution of chemicals and salts where the membrane maintains an ion concentration gradient between the intracellular and extracellular spaces. It is this electrical gradient that creates an electrical potential difference across the membrane and thus the cell membrane has electrically insulating qualities which is also known as capacitance.

On the other hand, as was discussed in the previous section, at low frequency range, it is mainly the conduction currents, which are dominated by freely moving ions, that is applied to the cell substrate and media and thus current flow only occurs on the cell surface by bypassing the cell membrane. The decrease of percentage viability of DLD-1 cancer cells indicates an increase for the number of dead cells where those cells lose the integrity of its membrane. The equivalent capacitance of cell substrate is dominated by

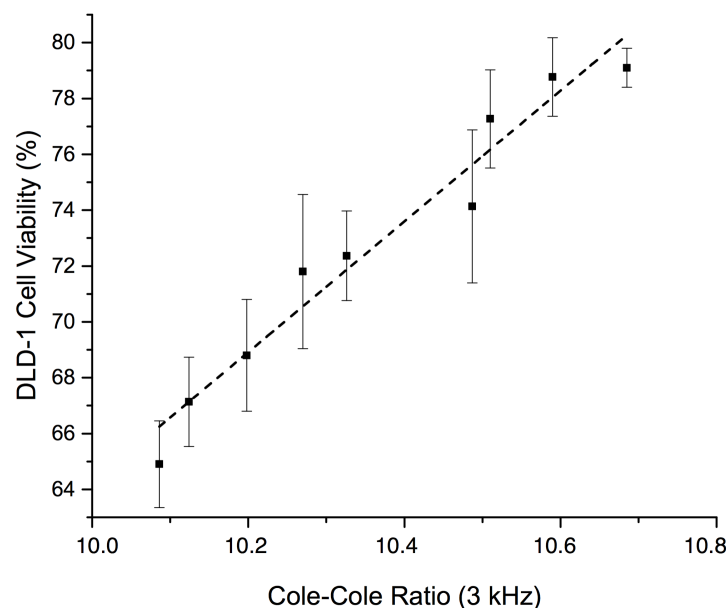


FIGURE 3.45: Correlation between Percentage viability of DLD-1 cancer cell and ratio of Reactance over Resistance of cell substrate at 3 kHz . ($R^2=0.957$)

the membrane. As membrane loses its integrity, it effectively removes capacitors from equivalent circuit model which includes both series and parallel connections. The equivalent capacitance, C_p , which represents the overall capacitance for parallel connected capacitors, is expected to be reduced if capacitors were removed and hence it increases the capacitive reactance; meanwhile the equivalent capacitance, C_s , which represents the overall capacitance for series connected capacitors, is expected to be increased if number of capacitors were reduced in series connection and hence decreases its capacitive reactance. The cell culture was implemented in monolayer microenvironment and membrane of cells were regarded as series connected capacitors. Therefore, the overall capacitive reactance will decrease as the percentage viability decreases at the α dispersion frequency range but does not follow the exact linear correlation. Meanwhile the breakdown of membrane integrity offers more pathways for ions to flow through intracellular substrate and the conductivity of cellular substrate is therefore increased and hence the overall resistance decreases proportionally with respect to viability percentage. Thus, the ratio between resistance and reactance at an α dispersion frequency range certainly has a more sensitive and accurate response to the percentage viability compared to the magnitude of Bioimpedance of the DLD-1 cancer cell substrate and it also contains more information regarding the cell substrate than bioimpedance.

These tests demonstrate the feasibility of using the in-situ bioimpedance measurement to determine the cell viability in the cell substrate. Further testing is required to identify the electric effect that a β dispersion ranged frequency could introduce. Testing of the sensitivity, reliability, sustainability and reproducibility of this portable chip-based bioimpedance measurement system is also essential. The direction of measuring current and the shape of electrodes also requires further investigation, as the conductivity along the direction of the cell substrate is stronger than the conductivity when measured perpendicular to the cell substrate.

3.4 Summary

A portable chip-based microfluidic platform for DLD-1 cancer cell viability determination was designed and fabricated based on the Bioimpedance Spectroscopy theory and utilising the reduced Electrical Impedance Tomography technique. The developed in-situ monitoring device on microbioreactor platform was constructed based on four sections, which are PDMS-based culture chamber, a miniaturised electric-impedance analyser chip AD5933EBZ, a multi-electrodes based data collection system and an analogue front-end (AFE) circuitry to enable the bioimpedance measurement for AD5993 chip. The Cyclic Voltammetry analysis identified the consistency of Pt for biological inspection sensing application. The proposed analogue front-end circuits were analyzed and concluded that Improved Howland Circuits exhibit most stable output current for cell monitoring. After reaching full confluence, cancer cell line DLD-1 was loaded into the PDMS microbioreactor at room temperature for 24 hours. The percentage viability were counted on the haemocytometer grid with Trypan Blue solution under fluorescent microscope observation. As the averaged viability of inspected cell sample drops from 79% to 65%, a number of bioimpedance measurements was simultaneously implemented. Results demonstrated the feasibility of replacing the professional, bulky-sized impedance analyser by utilizing an electric-impedance chip, AFE circuitry with simplified EIT data acquisition system. A reasonably linear correlation ($R^2=0.831$) between the haemocytometer cell viability and its bioimpedance measurement, with 3 kHz excited current, was illustrated. More importantly, a significantly better linear trend ($R^2=0.957$) was discovered by correlating the percentage viability of the DLD-1 cancer cell and Cole-Cole ratio at 3 kHz. The results show that the proposed technique provides an alternative and efficient method to monitor the cells viability in an controlled physicochemical environment during cell culture process compared with the conventional cellular analysis techniques, and this may have a potential application to monitor and control the cell differentiation process.

Chapter 4

Realization of An Automated System to Characterize Corneal Collagen I Scaffolds

4.1 Introduction

In tissue engineering applications and particularly in 3D cell cultures, the biological cross talk between cells and the scaffold is controlled by the material properties and scaffold characteristics. In order to induce cell adhesion, proliferation, and activation, materials used for the fabrication of scaffolds must possess requirements such as intrinsic biocompatibility and proper chemistry to induce molecular biorecognition from cells. On the other hand, collagen scaffolds can be derived from decellularized tissue and have been found important clinical application such as skin substitutes, blood vessels and engineered cornea. Corneal scaffolds are of special interests as the arrangement of collagen fibrils is critical to the transparency of the products, which is the key requirement. The microstructure is also important to the cell adhesion, penetration and repopulation of cells in the reconstruction of human cornea.

It is to be noted, as discussed in previous chapters, the concept of biocompatibility is referred to the ability of biomaterials, including polymers, electrodes and collagen scaffolds, to perform its desired function to generate the most appropriate beneficial

cellular response and ultimately to accommodate and facilitate the long term 3D cell culture in microbioreactor, without introducing any undesirable local or systemic effects in the recipient or beneficiary to that cell culture.

Among five different layers of cornea, which includes epithelium layer, bowman's layer, stroma layer, descemet's membrane and endothelium layer, stroma layer is said to be the most important layer as its material, known as collagen I fibrous, comprises 90% of total material of entire cornea [Tufekci et al. 2016; Shah et al. 2008; Lee et al. 2001]. Past literature also revealed that the corneal stroma is unique in having a homogeneous distribution of small diameter 25-30 *nm* fibrils that are regularly packed within lamellae and this arrangement minimizes light scattering permitting transparency [Land 1999]. It was hypothesised, based on the physical properties of light, that this lattice-like structure produces minimal light scattering and therefore, transparency [Maurice 1957] and this theory enables the feasibility of employing optical method for quality assessment of collagen I corneal scaffold.

Furthermore, the mathematical demonstration and correlated expression between scattering light and corneal collagen stroma transparency was developed by Benedek et al. (1971) and was experimentally proved by Farrell in the following years in 1973. The term transparency was defined as the percentage of transmitted light for each wavelength and it was found the transparency of corneal collagen is majorly dependent on four factors including i) thickness of the inspected material [Unchino et al. 2011; Lin et al. 2004; Cox et al. 1970]; ii) water inside of corneal collagen; iii) fibrils arrangement inside of collagen [Fraztl 2008] and iv) the abnormal accumulation of other materials [Hassell et al 2010].

Considering the unique application and characteristics of cornea-based collagen scaffold, in terms of transparency, and the potential risk of contamination in an open space, the collagen scaffold can only be inspected non-invasively via optical methodologies. A

number of direct and indirect detection methods was proposed and implemented. In early 80s and 90 the transparency of stored corneas was assessed directly with devices that measure light transmission through the cornea [Shashar et al. 1998] or with a patented optical device that is able to measure light transmission and diffusion of an ocular medium [Timothy 1997]. A few indirectly inspection methods was proposed in recent 15 years including a dark field biomicroscope and an optical coherence tomography, which is able to detect stromal scars [Edelstein 2016; Efon et al. 2002; Merin et al. 2001] and a device developed by the European eye bank [Jones et al. 2012]. Recently, the feasibility of optical inspection for cornea scaffold includes prototype investigation based on modulation transfer function analysis for a ratio of local contrasts [Accquart et al. 2013; Zheng et al. 2012] and development of lux-meter based detachable inspection system [Parekh 2013; Ventura et al. 2005].

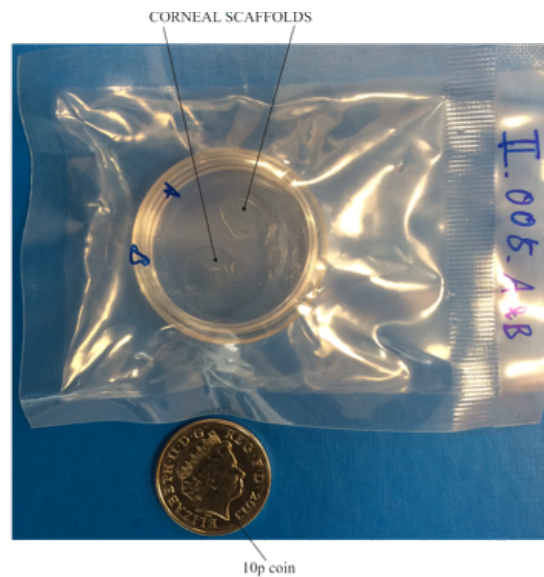


FIGURE 4.1: (a) Decellularized Corneal Scaffolds in a Petri Dish sealed by Polyethylene with a 10 pence coin to illustrate dimensions

However, none of above mentioned optical prototypes has been placed into daily practice at eye banks to objectively measure the optical properties of corneal collagen. Most

importantly, the inspected corneal collagen scaffold was exposed to air during the detection which risks contamination. Moreover, neither the correlation between the optical property and biocompatibility for corneal collagen scaffold, nor the non-destructive characteristics of inspections was discussed in the previous studies. More importantly no studies were trying to link microstructure and biological properties (e.g cell adhesion and cell penetration) with optical property. It is very possible that some of high energy based inspection itself could damage the collagen inner structure. None of above mentioned research implemented any biological experiments with inspected corneal scaffold, and hence has no proof for its biocompatibility with the cellular substrate. Thus, a new a non-invasive and non-destructive inspection system is demanded to not only identify the crucial optical properties for collagen scaffold, but also to find the link between collagen optical properties and its biocompatibility.

The provided corneal collagen scaffold sample was located un-specifically by the CRMI (China Regenerative Medicine International Limited) and was decellularized through physical chemistry methods and was sealed in a 42mm petri dish container, which is vacuumed by a plastic blister material(mainly polyethylene) measuring $80 \times 100 \text{ mm}$. The sample is illustrated in Figure 4.1; two scaffolds are sealed in a petri dish. However, a newly manufactured package with only one scaffold per petri dish was used during the verification and validation process.

The proposed optical inspection method, which is illustrated in Figure 4.2, involved the determination for the level and area of distribution of the transmitted parallel light through the decellularized collagen corneal scaffolds, and through the plastic material. The setup (i.e decellularized collagen corneal scaffolds and plastic material) can be affected by a number of properties when a parallel beam of light strikes the transparent specimen, such as the inhomogeneity of material, its thickness, smoothness and roughness of the surface, and wide-angle scattering.

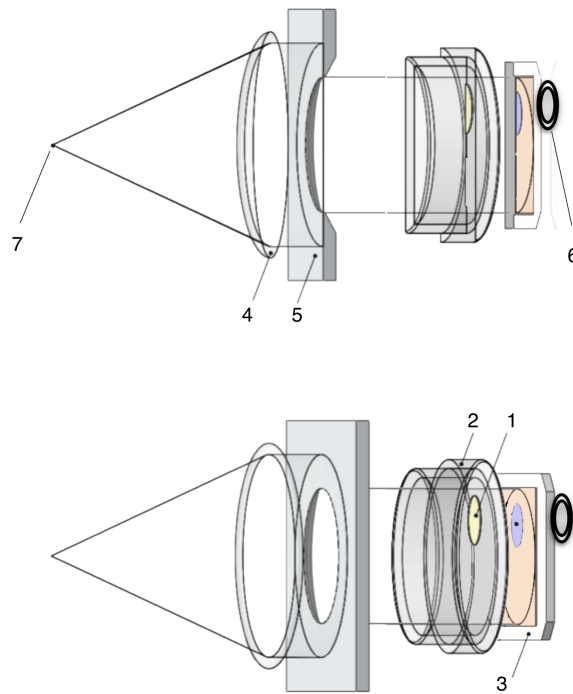


FIGURE 4.2: Proposed Principle Setup for Corneal Collagen Scaffold Inspection 1 - un-specified scaffold, 2 - polyethylene petri-dish, 3 - light beam after passing through sample package, 4 - Lens, 5 - knife edge stop, 6 - 7 mm aperture, 7 - luminosity source

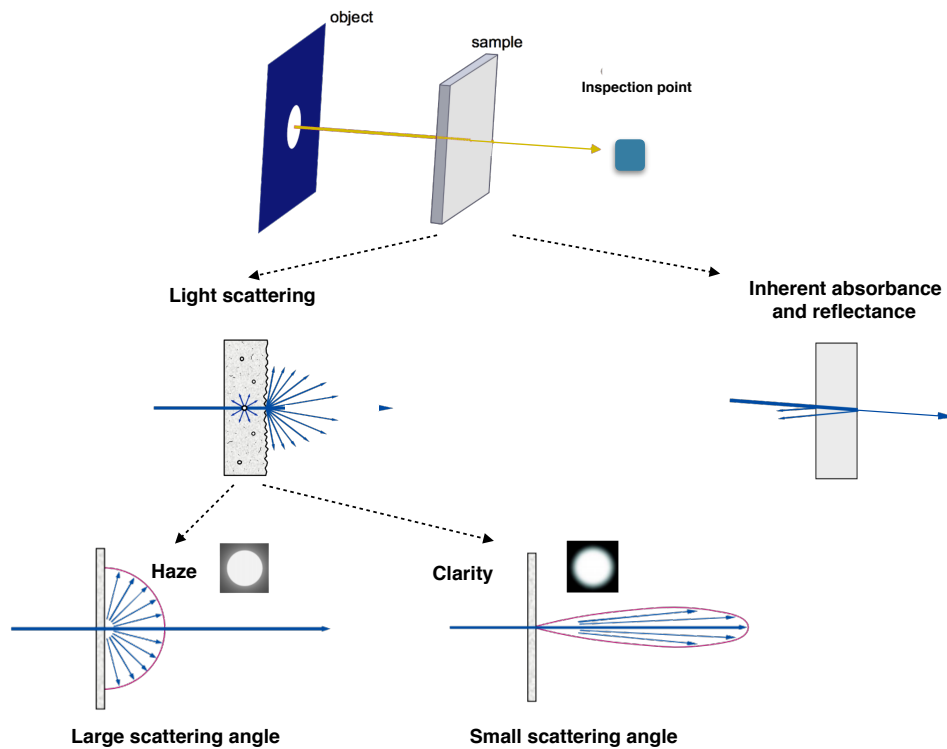


FIGURE 4.3: Relationship between different Optical Properties

Previous literature suggested the intensity of transmitted luminosity is reduced significantly by the inherent absorbance and reflectance of transparent material surface [Hassell 2010]. The amount of light that passes through the specimen unaltered is largely dependent on the surface smoothness and the ratio between total transmitted light and the incident light, which is defined as total transmittance [ISO DIS 14782 1999, refers to Web-List]. The quantitative amount of scattered light is defined by the particles (known as scatterers) inside the material. Its distribution is linearly linked with the scatterers size. If the parallel beams of light are scattered in a wide angle, i.e greater than 2.5 degree, where the light intensity per angle is small, the contrast of the image is said to be cloudy and is defined as haze. The increase of inspected material's depth will also increases the likelihood of light being scattered by particles inside the material and hence expected to reduce the light transmission rate and simultaneously increase the haze value. In contrast, if the light is scattered at a narrow-angle, the contours may be distorted, resulting in an image with less resolution; this is defined as clarity. The above mentioned relationships between different optical properties are illustrated in Figure 4.3. The hierarchy diagram for total transmittance and other related optical properties is summarized in Figure 4.4. It is to be noted that the level of clarity is dependent on the distance between the specimen and the observed object, which is beyond the scope of corneal scaffold material inspections. According to the EU standard ASTM D 1003 (ISO 13468), haze is the percentage of transmitted light that deviates from the incident beam by more than 2.5° on the average.

An improvement in accuracy was obtained by designing and integrating an automated system that positions the inspected material at the sphere inlet, which reflects light from the corneal scaffold within the sphere [Timothy 1997]. This improves the sphere efficiency and results in a more accurate total transmittance. An additional 7mm aperture was added to the optical inspection system. A vision recognition system, that fully cooperative with mechanical system, was also investigated and realized.

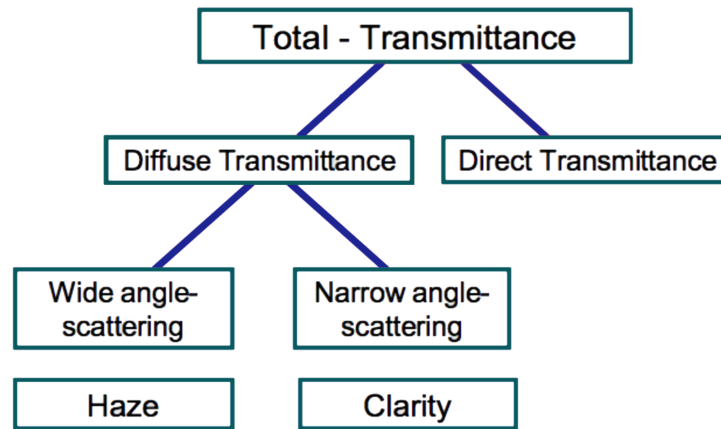


FIGURE 4.4: Hierarchy Diagram for Optical Properties of Total Transmittance

With these enhancements, the smoothness of the collagen surface will affect the adhesion process for the cell to attach to the scaffold. Inhomogeneity inside of the material will introduce incompatibility between cellular substrate and scaffold material, and will result in a reduced growth factor rate for the seeded cell [Kara 2011]. The nature of the material, such as the smoothness of its surface, can be indirectly determined by measuring parameters such as haze and total transmittance through high-precision optical instruments, e.g. Haze-Gard-plus (BYK Gardner Ltd).

Other than haze and transmittance, the pore distribution, surface area and porosity also play a major role; the amount and distribution influence the penetration and rate of penetration of cells within scaffold volume. Depending on the fabrication process, scaffolds with different architecture can be obtained, with random or tailored pore distribution. Thus there is a need to utilize the state-of-art technology to investigate the porosity, structure and purity of collagen scaffolds.

Those parameters were investigated via the state-of-the-art technology Multi-photon microscopy(MPM) was developed and realized based on the principle of second harmonic generation (SHG) in early 2000. The upright MPM (Nikon E600FN) not only provided the smoothness and roughness of the materials surface, but was able to image turbid

material in 3-dimensions to determine the purity and homogeneity inside the inspected transparent material.

In theory, the MPM employs pulsed infrared lasers that generate NIR photons for non-linear excitation of UV-excitable fluorescent molecules. Non-linear excitation requires the simultaneous absorption of two or more NIR photons that equal in energy of a single UV photon as illustrated in Figure 4.5. Thus, fluorescence outside of the focal volume is reduced during image acquisition allowing improved depth discrimination, submicron resolution, better image contrast and reduced out-of-focal volume bleaching.

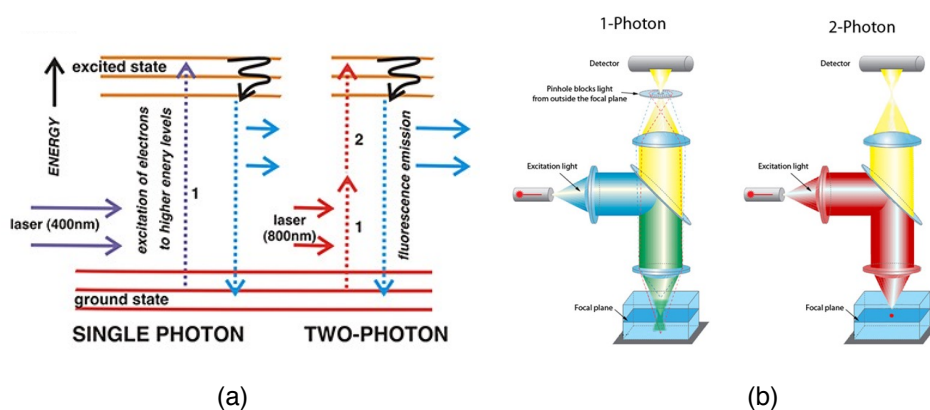


FIGURE 4.5: Illustration for relationship between different optical properties. Image from On-Line, refers to Website-list

Furthermore, unlike conventional microscope, the grayscale levels of MPM (SHG) are created not from counting photons that are not absorbed or scattered by the sample, but instead by counting photons that had their wavelength shifted (from infra-red to blue/green in the case of collagen), and filtering out all the rest. A large number of photons is therefore required because only some of them will get converted and the material that does the conversion must have a special regular repeating structure that has a periodic pattern but is not a regular centrosymmetric crystalline structure. In fact Second-harmonic light emerging from an SHG material is exactly half the wavelength (frequency doubled) of the light entering the material.

On the other hand, the periodic repeating pattern of collagen is very similar to a crystalline structure and more importantly the exact 67nm spacing of that structure leads to reflect back enough SHG wavelength with double frequency so we are able to observe the effect. Other proteins that do not have repetitive structure will not resonate in way that reflected signal was not strong enough for observation. As a result, by utilizing MPM for type I collagen inspection, it is expected to get very high instantaneous photon count, but still keep the average power level low enough to avoiding vaporise the sample.

Although MPM has the power to detect the structure inside of biomaterials, it has limitations: (i) complex to operate and maintain; (ii) only a small area of $500\ \mu m \times 500\ \mu m$ can be identified in a single scan; (iii) each scan takes 20 - 30 mins and is unsuitable for high-throughput screening. Furthermore, due to the system setup (up-right microscope), the cell culture inside of PDMS culture chamber cannot be inspected.

Thus in this thesis, the MPM was mainly only used as an assistive tool to investigate ultrafine structure with material purity and homogeneity at specific location inside of type I collagen scaffold since according to Nakayasu et al. (2004) and Tsunoda et al. (2001) different layers of collagen inside the stroma may present different patterns. The image reconstruction was implemented in Imaris software which allows the user to slide through the scaffold in vertical-axis with pre-defined signal strength in RGB and to determine the conditions of different collagen layers. The Isosurface was used in MPM to construct a surface (and volume) for collagen signals that has equal or higher signal intensity from the selected threshold. The operational difficulty is to select an appropriate threshold to collectively reflect the collagen structure by accurately determining the upper and lower edge of scaffold (same one used to determine scaffold thickness).

By designing and integrating an automated non-invasive inspection system, its expected to eliminate human operational errors and enables high-throughput screening quality

control. It is expected that the proposed methodology is non-destructive to inspected materials, and will not affect the cell culture after inspection. The developed system was expected to facilitate the understanding of how cells interact with type I collagen scaffold, and will indicate material biocompatibility with cellular substrate that contribute to increase success in clinical transplantation which is explained in the next chapter.

It is noted there are alternative non-invasive and non-destructive inspection technologies available which would be of great value to investigate. A number of pioneering feasibility studies were implemented prior to the design that demanded automation system for precise optical inspections, including a collimated beam and polarised beam with CCD array detection, Electrophotometry and Line-scanner(TSL1401), Single Pixel Luminosity Sensor (SPLS) and Haze meter. However, the purpose of this project is to design and realize a reliable automation system to provide accurate optical information and hence indirectly indicate its biocompatibility. While it was implemented from scratch under a very strong time constraint, it was in hindsight a rational decision to reduce customized digital interface with existing commercially available machinery while intensively collaborating with third-parties; prioritizing the implementation and integration of more fundamental parts of various detection systems, the decision was made to not realize self-developed Line-Scanner system as the time was very limited. However, while recognizing its relatively incisive nature, the current system should be acknowledged for its significant contribution to the future development of a non-invasive real-time quality control system for decellularized Biomaterials. Overall, forty-six samples were assessed for purity and homogeneity via MPM and ten scaffolds, excluding those identified to be impure, in the feasibility studies. Furthermore, forty samples were inspected via the newly fabricated automation system three times, followed by statistical analysis.

To minimise the human operational errors to a controlled level, the design principle for an automation system, that would cooperated with selected inspection methodology,

was discussed at initial stage. The sub-section of automation system includes XZ motorized mechanical system, a vision recognition system, analyzing system and interfacing unit which were investigated separately and later integrated to one system, followed by assessments for its reliability and repeatability. The collagen corneal scaffold was then investigated via the fabricated automation system and the precisely recorded data was statistically analysed to identify the selection bands for biocompatibility. The final investigation was carried out with MPM to investigate the local ultrafine structure combined with 3D reconstruction in Imaris software to verify the type I collagen purity and homogeneity and try to establish the link between local ultrafine structure to quantitatively identified Haze parameter.

4.2 Design Principle of Optical Systems and Identification of Key Parameter

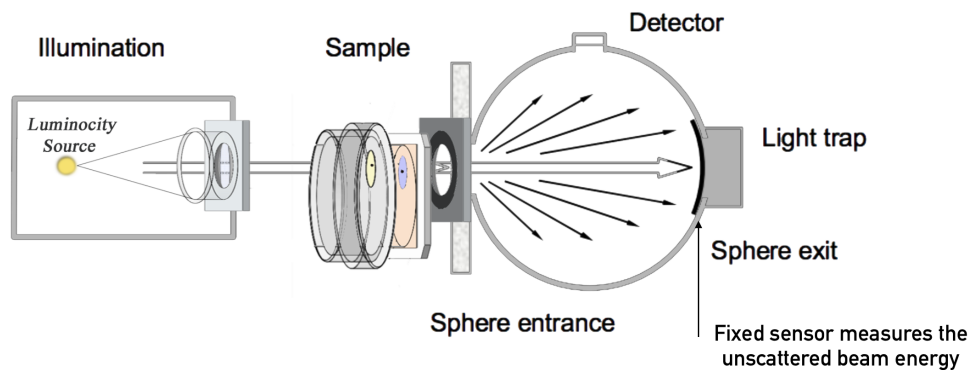


FIGURE 4.6: Illustration of Design Principle for Total Transmittance Measurement Set-up

A number of pioneering feasibility studies were implemented prior to the fabrication of integrated system. These include a Collimated beam and polarised beam with CCD array detection, Electrophotometry and Line-scanner (TSL1401), Single Pixel Luminosity Sensor (SPLS) and optical Haze meter. It was found that the optical Haze meter is able to offer a quantitative analysis with highest reliability among the alternatives.

The concept for utilizing the existing Haze-Gard-plus (manufactured by BYK Ltd) meter, as a stationary instrumentation to quantitatively identifying the quality and appearance of transparent materials include decellularized collagen corneal scaffold and polyethylene materials, was manually examined with 40 unique corneal scaffold samples (excluding the six samples with impurities). The experimental setup is illustrated in Figure 4.6 and Figure 4.7. A simple luminosity source, with spectral sensitivity conforming to CIE standard, provides paralleling beams collimated with an optical lens. The specimen surface is illuminated perpendicularly to the paralleling beams and the transmitted light is then measured photoelectrically via a spherical shaped sensor (0° /diffuse geometry).

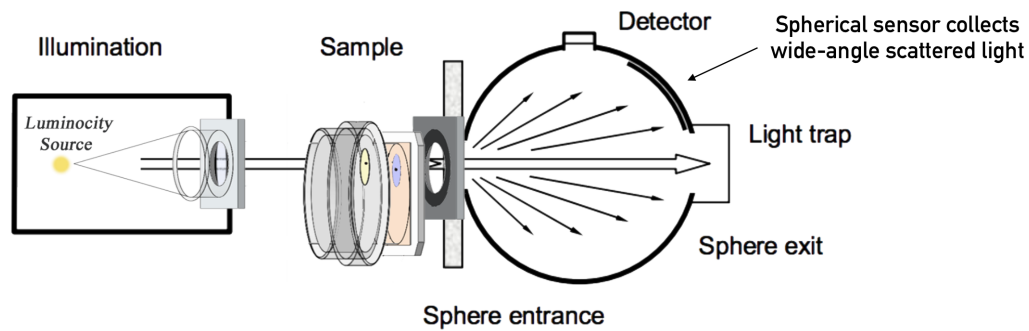


FIGURE 4.7: Illustration of Design Principle for Haze Measurement Set-up

An additional 7mm aperture was added to the optical inspection system. Reducing the aperture of the system from 18 mm to 7 mm effectively decreases the luminosity gathering area by 61%, provided the lens focus length remains unchanged. This not only contributes to reducing the distorting luminosity from background light, but also ensures that the entire transmitting area is only covered by corneal collagen scaffold, thereby increasing the systems accuracy and reliability.

Despite the improvement in luminosity by the 7 mm diameter aperture, a percentage error of $\pm 23\%$ and $\pm 17\%$ in haze and total transmittance value were measured, which are considered to be high for precise optical inspection. This is because the

diameter of the central area of the corneal scaffold lies within the range between 8 *mm* - 10 *mm*. Thus to obtain accurate haze and transmittance values, the central area of corneal scaffold needs to be positioned precisely with the inspecting light beam. This is practically difficult to achieve for large number of corneal samples inspection where human operational errors are inevitable. Therefore, an automated system with significantly reduced operational errors is needed for collagen corneal scaffold inspection.

4.3 Development of An Automatic System

In order to command the automated system for non-invasive and non-destructive corneal scaffold inspection, an integration of existing units was proposed and the top-level design of the proposed system is illustrated in the Figure 4.8 and the proposed diagrammatical system assembly is shown in Figure 4.9. Three sub-automated-systems were implemented in parallel: the analyzing system, vision recognition system, and mechanical system. Each systems reliability and repeatability were tested and verified over a large number of repeated operations. The verified systems were then integrated at a later stage.

The above optical arrangement was intended to be a component of a serial inspection system with a keep or reject principle applied to the corneal scaffold samples based upon the aforementioned light transmission quality. The manual switch for haze meter was set to be dysfunction and lately replaced by a programmed digital signal transmitter through double parallel point (known as DB17) connection. The haze-Gard-plus also transmits with PC via I2C protocol and outputs the real-time recorded data into the desired formatted file whenever measurement took place.

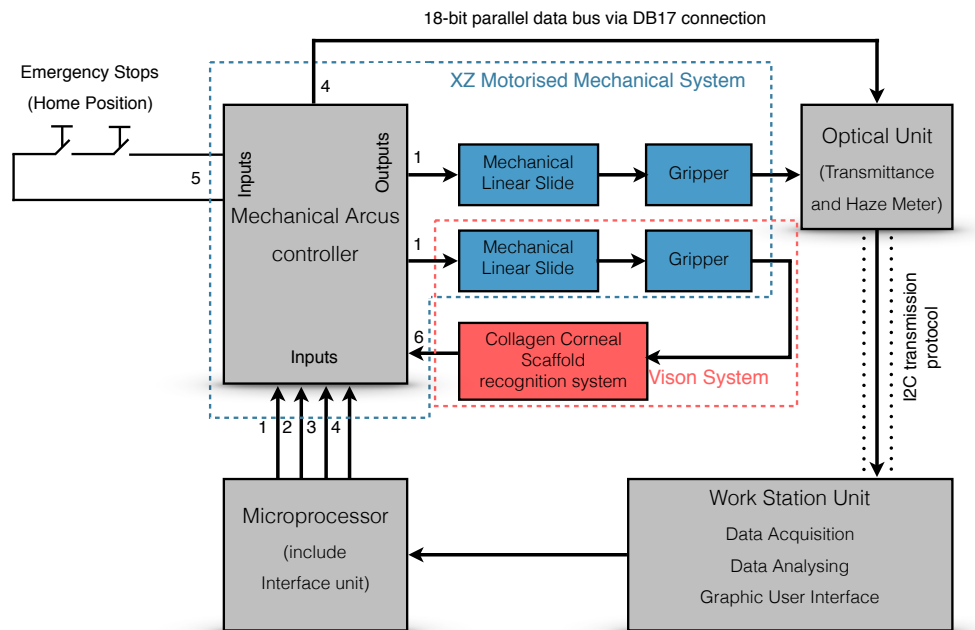


FIGURE 4.8: The proposed Programmatic and Functional Schematics

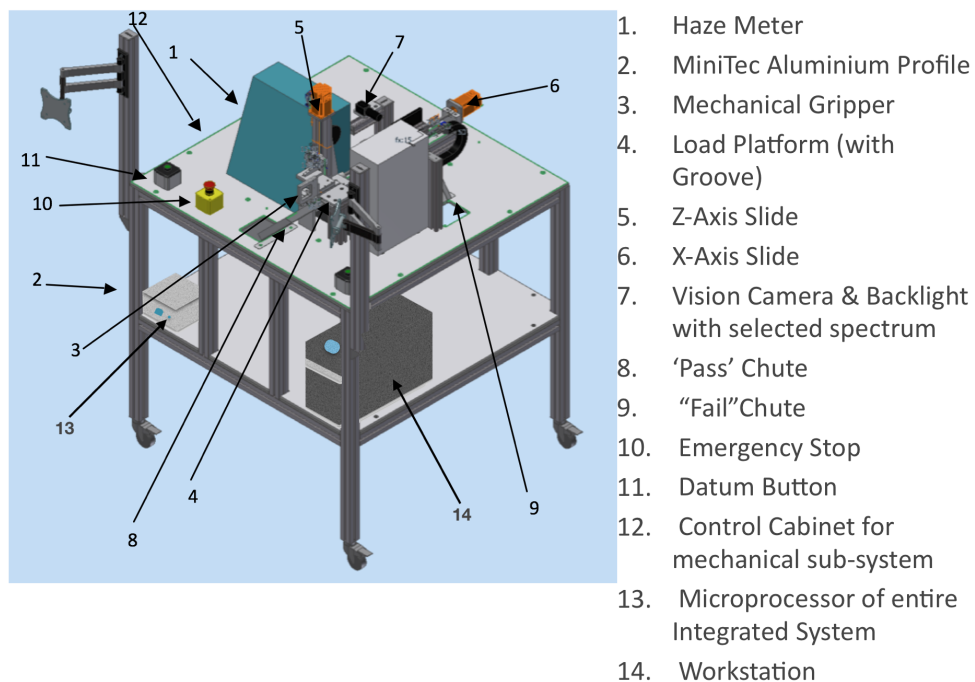


FIGURE 4.9: Diagrammatical System Assembly

4.3.1 Mechanical and Vision System

The mechanical system was realised by utilizing the stepper-driven based ball screw platform with single linear motion guide bearings that is controlled by the micro stepping processor, Arcus PMX-4, which quickly synchronises inputs and outputs for the coordination of motion and other operations during inspection. It is important to clarify that the overall system design and control algorithm of the automated system was proposed by me. During the system fabrication and integration process, majority of sub-systems include the communication between workstation and Haze meter, the interface unit between workstation and mechanical system, the development of GUI, the data acquisition and analyzing system were all realized and testified independently by the author of this thesis, whereas the realization of XZ motorized stepper driver was fabricated and provided by LG Motion Ltd. A close collaboration was carried out in parallel with an imaging specialist team Scorpian Vision Ltd to develop a feasible pattern recognition system and its interface to mechanical system. Nevertheless, the investigation of collagen corneal scaffold recognition rate under different spectrum was investigated independently by the author.

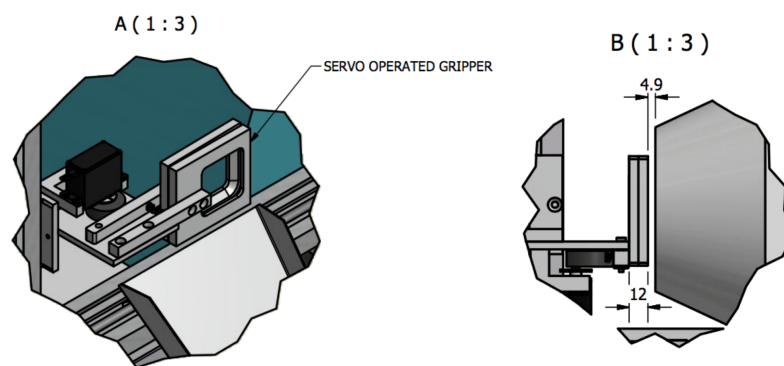


FIGURE 4.10: Illustration of proposed mechanical Gripper (scale 1:3)

It is advised by the haze meter manufacturer that precisely positioning the inspected material at the sphere inlet will result in a more accurate total transmittance. Thus, brackets and a fixture for identification of the centre of corneal scaffold was realised,

along with the gripper mechanism to hold the sample firmly in place. The optimal location for inspection is set to be perpendicular to the luminosity source and 4.9 mm distant away the exactly sphere inlet as illustrated in the Figure 4.10.

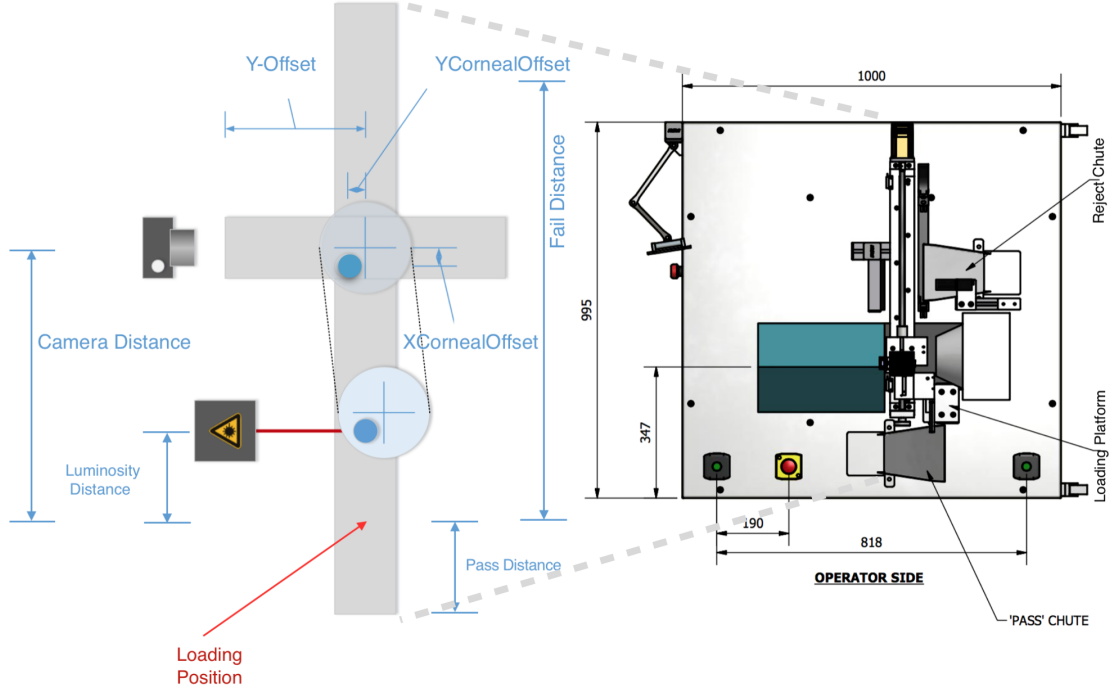


FIGURE 4.11: Top Side View of Proposed Automation

The stepper-driven mechanical arm held the loading sample and moved along the stepper platform with Camera Distance, D_{camera} , as specified in the 'Settings'. The programmed vision system is then able to identify the offset distance known as X_{Cornea}^{Offset} and Y_{Cornea}^{Offset} from the centre. This centre is based on the 'Luminosity Distance', $D_{Luminosity}$, and 'Y-Offset' that was specified in the pre-setting database as illustrated in Figure 4.11. The vision system is set to fail if the centre of the corneal scaffold is unrecognised and the mechanical arm is then automatically returned to the sample loading location and waits for 20s. Otherwise the gripper will bring the inspected sample by the calculated distance.

$$X = D_{Camera} - D_{Luminosity} - X_{Corneal}^{Offset} \quad (4.1)$$

$$Y = Y^{Offset} - Y_{Corneal}^{Offset} \quad (4.2)$$

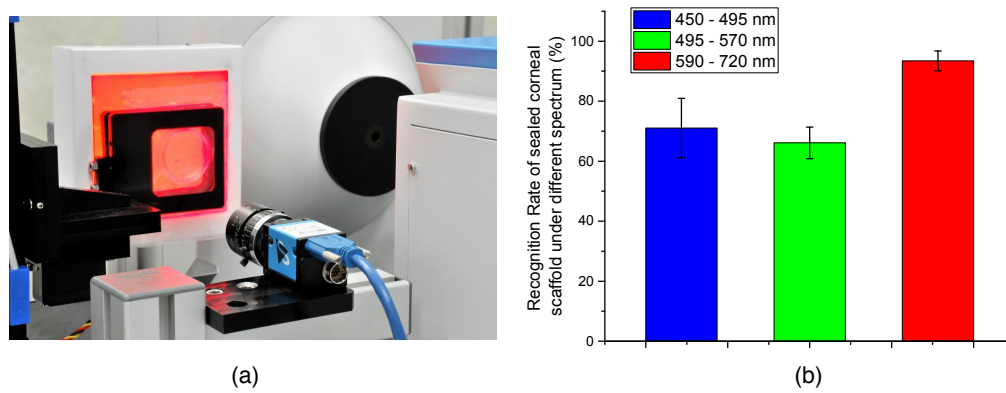


FIGURE 4.12: (a) Demonstration of Vision recognition system for identification of corneal centre in petri-dish under a sealed condition; (b) Recognition rate of corneal scaffold for its centre point under different spectrum

Collagen scaffolds were suspended in a petri dish without specific locations, making it difficult to determine the exact position with relation to the packaging. First, a multi-stage algorithm, Canny edge detection operator, was employed in vision recognition system to identify the edge of the collagen scaffolds. During the vision recognition process, the embedded codes find the intensity gradient at each pixel and quantize into one of four following directions: north-south, east-west, northeast-southwest and northwest-southeast. It is considered to be a candidate edge point if the magnitude of gradients is greater than the two neighbouring pixel along the above-mentioned directions. The centre of the collagen cornea scaffold was then determined by utilizing the identified true edges. The proposed vision system was proved to be reliable and efficient, providing a good detection with minimized probability for false edge detection, a good localization that the detection are close to the true edges where only one point represented each true edge.

Furthermore, the feasibility of pattern recognition and centre identification under different spectrum was also carried out under the experimentally selected spectrum range. Figure 4.12(a) demonstrated the integrated vision sub system for corneal central identification. The results of scaffold recognition rate are given as mean $\pm$ standard deviation

of three repeated experiments ($n=9$) and results of 46 samples is shown in Figure 4.12 (b). The recognition rate were 93 % with ± 3.3 % standard deviation in orange/red spectrum range, which is significantly higher than the detection rate under blue, 72 % ± 9.9 %, and green, 66 % ± 5.3 %, spectrum range respectively.

The obtained F-Ratio through ANOVA statistical analysis with (2,6) degrees of freedom was determined to be 14.02 which is larger than the given critical value of F (=10.93) with significant level of 0.01. Thus the ANOVA analysis demonstrated there is a significant difference for corneal recognition in a sealed environment under different spectrum range and the 590 $\sim$ 720 nm wavelength range was discovered to be the most favourable environment for corneal centre identification.

After successfully validating the vision recognition system under different spectrum, the sub-system was integrated through Arcus PMX-4, followed by a large number of mechanical system reliability tests for 200 hours, with no major mechanical faults to be found. Three additional components were tested and added to the sub-merged mechani-

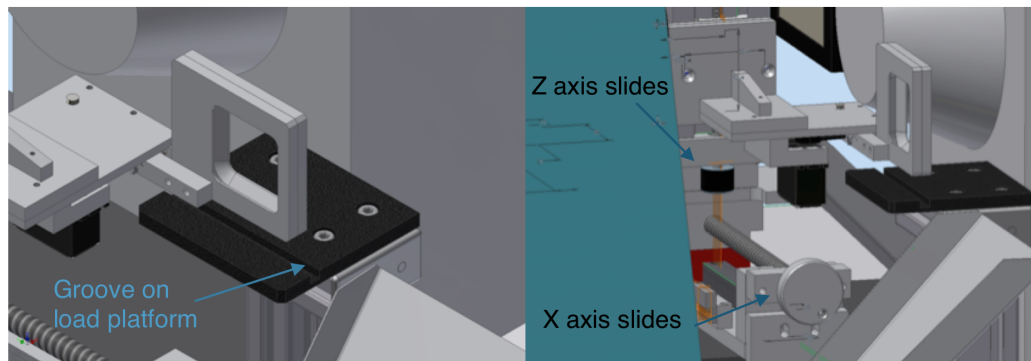


FIGURE 4.13: Demonstration of mechanical gripper with ± 5 degrees of self-adjustment (left) and manually adjustable motorized slides (right)

cal system; these were a groove in the loading platform, a manually adjustable motorized slides and two emergency stops for terminating mechanical movement. The inspecting sample is loaded on the loading platform, where the bottom of the plastic package is inserted into the groove; this holds the inspecting sample upright until the gripper closes

around packaging, as shown in Figure 4.13 (left). This ensures that the mechanical gripper holds the sample securely during the inspection process. The Manually adjustable motorized slides were added into the system as part of a resetting process either due to a failure in the control system and/or mechanical breakage. The turning of knurled hand wheels fitted to the end of X and Z slides as illustrated in Figure 4.13 (right). In addition, two emergency button were added to the design to shut-off the entire mechanical and vision recognition system under emergency circumstances. These buttons aid in avoiding damage to tubular components and/or operators, one of which was mounted directly in from of the operator position on the system work surface and the other one fitted to the front of the control cabinet.

4.3.2 Flow of Control

The control flow of sub-systems was designed in the early fabrication stage and is shown in Figure 4.14. The overall integration was implemented after the reliability tests of vision recognition system and mechanical system had been verified. The haze meter transmitted the recorded data to PC via I^2C protocol and wrote/generated the output data in the designated file in a specific format. The designated file was kept open during the measuring period and new data was added on the real-time basis. The data analysis system was then proposed and designed based on the principle that the system regularly (with interval of 20s) imported data from the haze meter's constantly-opened file, and stored the imported data in a temporary location in the PC. The waiting cycle for data acquisition was set to 20s as the whole operating length for both mechanical and vision system was 35s with systematic propagation delay of $\pm 3s$, provided the motorized speed was set to be default value (30 mm/s). The mismatch between data input and data output cyclic period (20s and 35s), was set to avoid potential clashes between these two operations. The cyclic importing period (20s) was set to lie between half of the operational period (17.5s) and complete operational (35s) period; this guarantees the

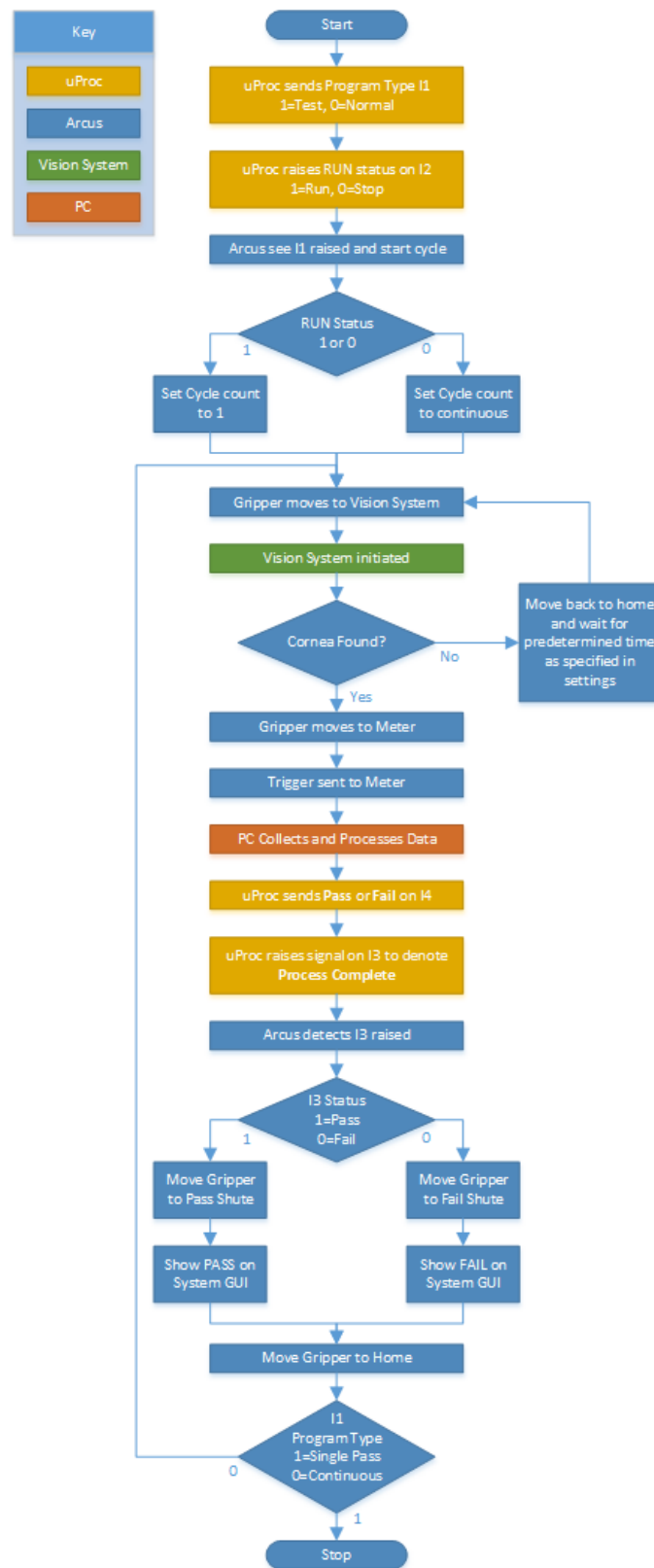


FIGURE 4.14: Control Flow for Fully Integrated Automation Inspection System

transmission of data from the haze meter. The imported data was identified for its matrix size (vectors) via a pre-embedded algorithm, which directly indicates whether any newly recorded raw data was transmitted into the system.

It was then analyzed based on the embedded algorithm, which is shown in Figure 4.15 (left) and (right), combined with experimentally determined thresholds for relevant parameters including haze and total transmittance, thereby resolving the optical property of collagen corneal scaffold non-invasively. The upper and lower limits of haze and total transmittance parameter for automated quality control were designed to have the flexibility for customised alterations through developed GUI, since the different patches of corneal scaffold tend to have different physical properties. The validation experiment demonstrates that the maximum amount of data that were successfully imported within a second was 4,480 numerical values and the shortest importing period through experiments without clashes to haze meter system is 5 seconds. This enabled potential applications of integrated systems for different needs in the future with higher mechanical movement speeds.

4.3.3 System Integration

The automatic measurement was controlled through a customized GUI front-end program that allowed simple system operation without the need to program. A number of programmed operations were embedded in the algorithm along with a display of its status such as measuring time, haze parameter and total transmittance for the inspected scaffold. The inspected data can be exported as an Excel file and permanently stored in a PC. The communication between inspection outcome from data analysis system and mechanical system was enabled by I^2C protocol based micro-processor Arduino ATmeg 328 and Staint Smart 8 switching unit. This enabled the mechanical arm to deliver the non-invasively inspected scaffold to the designated location (i.e pass bin or

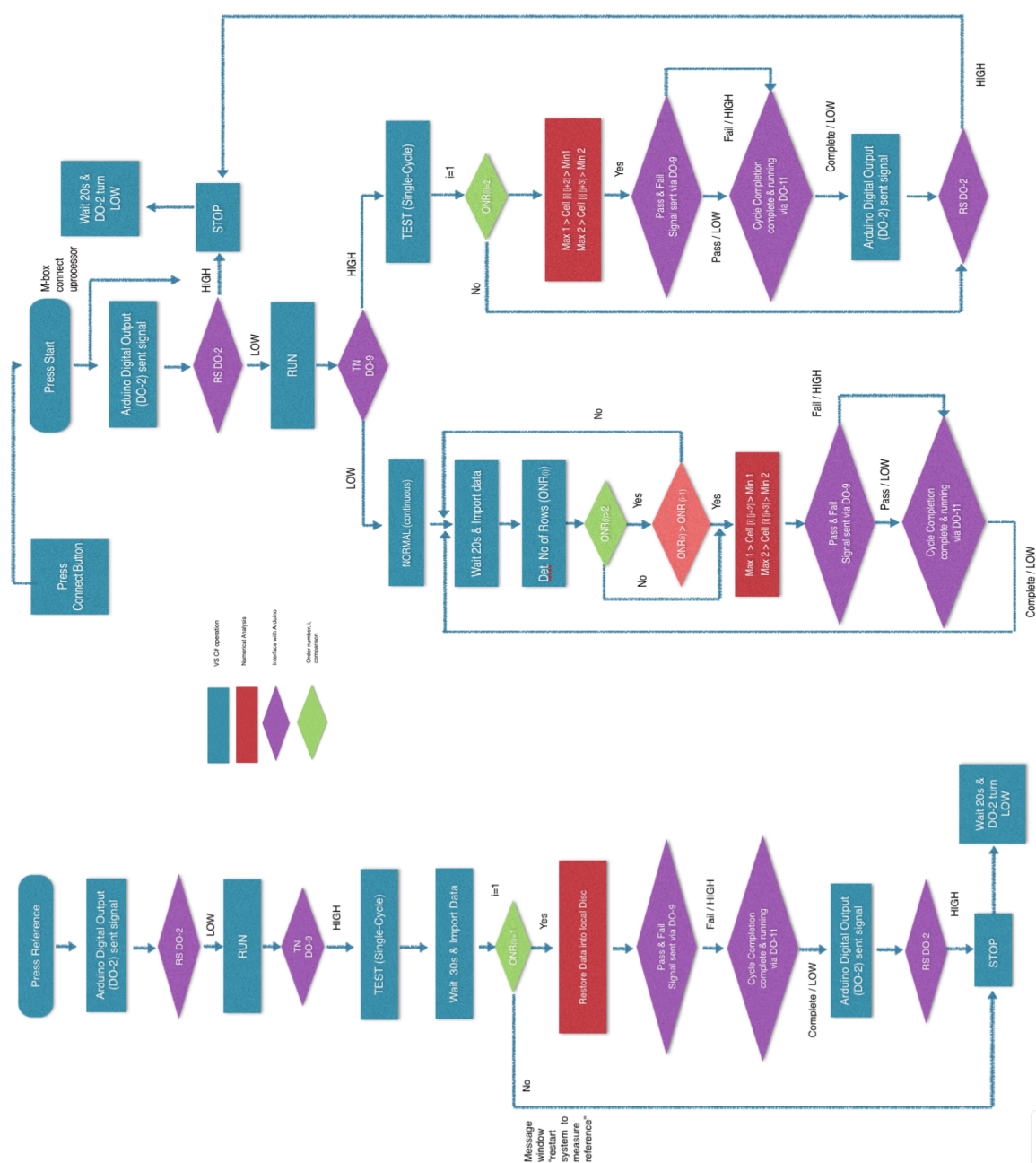


FIGURE 4.15: Control Flow for Haze and Total Transmittance Data Acquisition and Analysis

fail bin) with respect to inspection outcome. It is noted that the most common I/O operations for both mechanical and systematic processors are set to low; this not only reduces power consumption during communications, but also decreases the excitation current pass through switching unit, thereby reducing chances of an electronic fault occurring over long term operation. The interface processing unit was customized and firmly assembled in an aluminium enclosure platform (RS 746-7346) where all PCB and DIN rails were installed in the chassis plate. Furthermore, a large number of terminal blocks were used to improve the stability for data transmission and Nylon cable glands were also added for waterproof and insulation purposes.



FIGURE 4.16: Demonstration of fabricated and integrated system

Overall, the integrated system, as illustrated in Figure 4.16, was verified to automatically load corneal scaffold samples to the pattern recognition region, which located the centre of the corneal scaffold within $\pm 0.2 \text{ mm}$. The data was then sent back to the mechanical controller processor, Arcus, to reposition the scaffold to an optimal location that is perpendicular to the luminosity source and 4.9 mm distant away the exactly sphere inlet. Once the optimal position was reached, a feedback signal was correctly received at Arcus and correspondingly sent the digital signal through the replaced DB17

connection to activate the haze meter to take a measurement, followed by correct analysis and mechanical sorting. The data was then stored permanently and analyzed through numerical software. The system's reliability was tested for 200 hours collectively without any major fault.

4.4 Characterization of Corneal Scaffold Using the Automation System

The first target of the experiment was to determine the feasibility of a newly integrated and developed inspection system which is demonstrated in Figure 4.17. This was obtained by verifying the sensitivity of the system with 46 selected unique corneal scaffold (from single patch) that all sealed in 42 *mm* petri dish followed with data analysis which then differentiated samples into several bands.

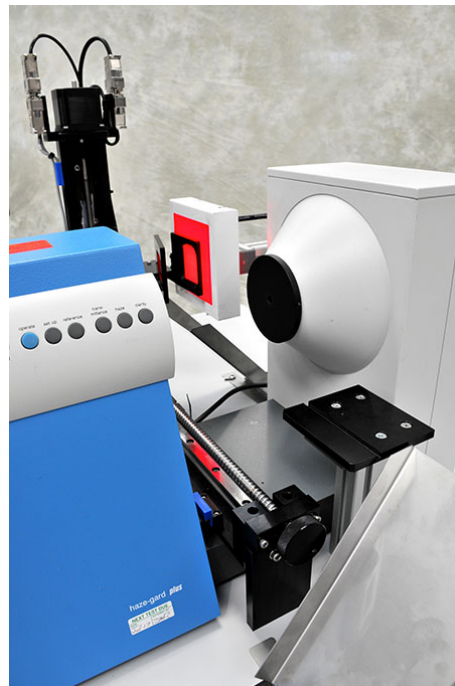


FIGURE 4.17: Demonstration of Fabricated Load Platform

Each sample was loaded and automatically examined to evaluate the reliability of the automated system. In addition, a reference measurement was taken for every single sample where only petri-dish and polyethylene plastic material were inspected. The scaffold optical parameter was non-invasively determined by subtraction between overall sample measurement and reference measurement. The measurements for corneal scaffold sample as well as reference for each sample package were repeated for three times separately and the relevant mean, mode, standard deviation values were calculated accordingly. Table 4.1 concisely summarises the collected data.

	Total Transmittance (%)			Haze (%)		
	Sample	Polyethylene Material	Scaffold Transmittance	Sample	Polyethylene Material	Scaffold Haze
Mean	63.36	62.71	99.35	49.93	33.91	16.02
Maximum	68.10	66.65	98.55	63.40	40.21	23.19
Minimum	60.50	58.77	98.27	32.20	22.40	9.80
Standard Deviation	1.63	1.54		4.21	4.30	

TABLE 4.1: Optical properties value includes Total Transmittance and Haze that obtained through inspection via newly integrated automation system. (Values displayed in the Polyethylene column is the corresponding value to the inspected optical properties)

From Table 4.1 only 1 % difference ($98.27 \sim 99.35$) exists between separately calculated maximum and minimum values for total transmittance by subtracting polyethylene measurements from package sample measurements. This reflect an excellent repeatability for an automated system operation and hence proves the system reliability. The absolute difference in total transmittance measurement between sample and polyethylene material is in the decimal range and comparatively negligible to the measured values. It is therefore sensible to assume the losses in total transmittance were majorly caused by the polyethylene material which surrounded the scaffold.

In contrast, the haze measurement revealed that both polyethylene material and scaffold contain haze parameters. The measured data over scanning of 46 samples most lay

between 9.8 to 23.19 and the smaller the haze value is, optically the better material it will be. Furthermore, measured data also suggests the haze effect caused by the polyethylene material is 2.3 times larger than the haze effect given by the scaffold, which is the dominant factor in the inspection system.

From high-throughput screening quality control perspective, it is practically difficult to collect all of the data from an entire population, instead, a number of subset of data (randomly collected samples) from an industrial process were gathered to make conclusions about the quality and reproducibility of samples. The central limit theorem (CLT) had been reportedly being utilized for quality control of industrial process since, by the nature of industrial process [Smith 1998; Moore 1996;], if the independent random samples (of size n) were repeatedly taken from any population, when n is large than 30, the distribution of the sample means should approach a normal distribution [Bryce 1989; Romano 1971]. It also indicate how far a set of sample are spread out from their mean.

Moreover, it is known that for the normal distribution, the values less than one standard deviation away from the average mean account for 68.27 % of the measured set, while two standard deviations from the mean account for 95.45 % of the overall samples; and three standard deviation accounts for 99.73 %. This is known as 68-95-99.5 empirical rule or the 3-sigma rule and it is illustrated in the Figure 4.18 and c) where a nonparametric kernel density estimator is added to each normal distribution to smoothly represent the distribution of recorded data with respected to the corresponding density at each region. This is regarded as a common tool in practical data analysis since the 68-95-99.5 empirical rule helps to understand where to expect the future data to range.

With respect to measured data, the total transmittance rate remains unaffected when the light beams pass through corneal scaffold non-invasively. Thus the dominant factor in total transmittance rate, for automated quality inspection purposes, will depend on the chosen range of tolerance interval under 3-sigma rule in the normal distribution.

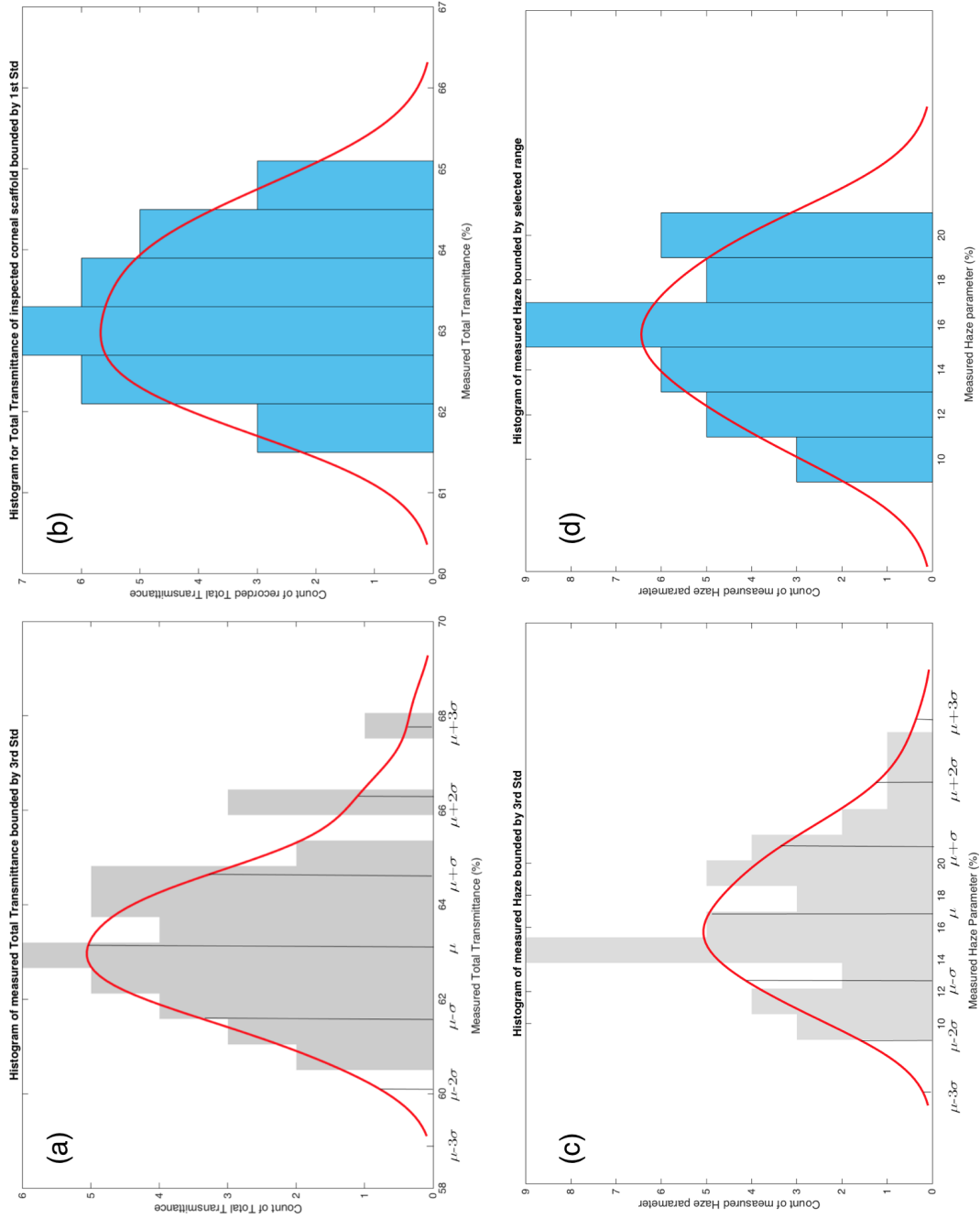


FIGURE 4.18: Histogram for obtained optical inspection data (a) Total Transmittance bounded by 3rd-Sigma; (b) Total Transmittance bounded by 1st-Sigma; (c) Haze bounded by 3rd-Sigma; (d) Haze bounded by selection band

	Total Transmittance (%)	Haze (%)
± 3-Sigma	58.47 ~ 68.25	5.86 ~ 25.30
± 2-Sigma	60.10 ~ 66.61	9.10 ~ 22.06
± 1-Sigma	61.73 ~ 64.79	12.34 ~ 18.82
Selected Range	61.73 ~ 64.79	0.00 ~ 21.10

TABLE 4.2: Determined boundaries for Total Transmittance and Haze parameter under 68-95-99.5 Empirical Rule

From a strict quality control perspective, the selected range should be no more than one standard deviation away from the mean and the normal distribution for measured total transmittance value within selected range is shown in Figure 4.18 (b). The distribution for total transmittance under 3-sigma conditions is slightly negatively skewed (mean > median), whereas the distribution in the newly selected range is more symmetric.

On the other hand, selection range for haze parameter was collectively determined based on the optical property of material and strictness of tolerance interval under normal distribution. Based on the principle of haze measurement, less haze implies higher light intensity per angle and smaller amount of light diffuses through scaffold with scattering, thus better contrast. Therefore, the lower limits of the selection band are set to zero and the upper limits are set to one sigma away from the mean. This should, theoretically, enable the system to keep 50 % of the inspected corneal scaffold sample under haze inspection. The accurate value for each interval is summarised in Table 4.2 and Figure 4.18 (d) shows the normal distribution for obtained haze data within the newly selected range.

Better statistical understanding can be achieved through the probability density function (PDF) which is shown in Figure 4.19(a)(c). The PDF curve of the total transmittance within the selected range appeared to eliminate the negative skewed characteristics compared with 2-sigma and 3-sigma and provide a better symmetric distribution. Comparatively, the PDF of the haze curve for selected range shares the same tendency with

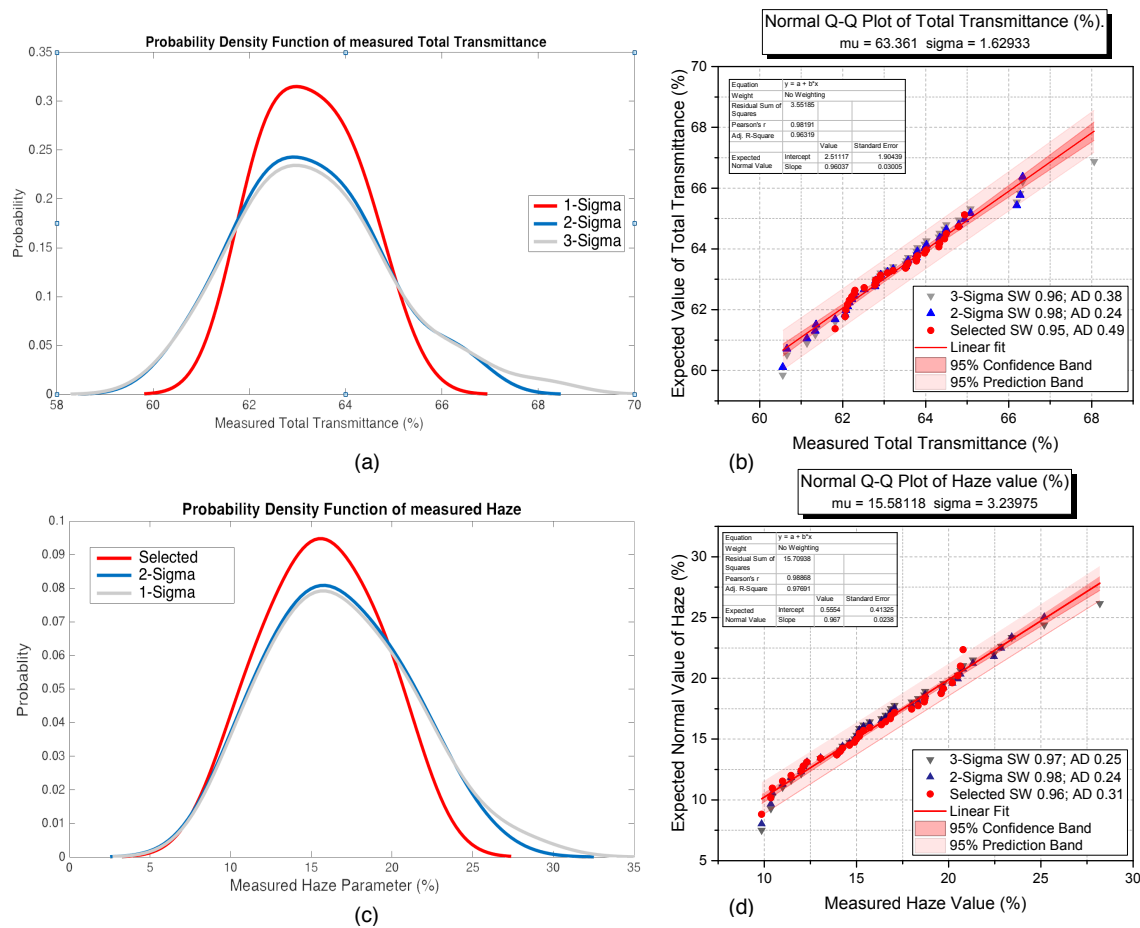


FIGURE 4.19: (a) & (c) Probability Density Function of measured data; (b) & (d) Quantile-Quantile plot of inspected data for corneal scaffold in the sealed condition. In each diagram Red curve and points represent the analysed data filtered by selected bands; Blue represent the analysed data filtered by 2nd-Sigma; Grey represent the analysed data filtered by 3rd-Sigma. Shapiro-Wilk test and Anderson-Darling test were abbreviated as SW and AD respectively in the legend and were used to identifying the normality of inspection data

2-sigma and 3-sigma curve, but provides steeper accumulation in probability density at higher boundaries of the selection range.

To verify the normality of measured data within selected bands, the normal Quantile-Quantile plot (q-q plot) of total transmittance and haze was generated and is shown in Figure 4.18 (b) and (d). The linearity of points between a randomly generated, independent standard normal data and experimentally measured total transmittance and haze suggests the data are normally distributed. Further statistical analysis includes Shapiro-Wilk test and Anderson-Darling test revealed that normality of measured data

within selected bands was improved compared with distribution within 2-sigma and 3-sigma bands and the corresponding p-values for both normality tests also indicate the measured data within selected range was significantly drawn from a normally distributed population at 0.01 significance level. The prediction band was also determined during regression analysis and it indicate the prescribed probability for the values of future haze and total transmittance measurements from similar population sample.

To conclude this section, separate continuous operations of the integrated automation inspection system reveals its excellent sensitivity and reliability. The measured optical data was statistically analysed to identify the most suitable selection band for non-invasive collagen corneal scaffold inspection. The selection band for both total transmittance and haze parameter was chosen independently through optical theory and the consideration of practical issues. The newly defined selection range was then embedded into the control algorithm for future inspection operation.

4.5 Multi-Photon Microscopy for Ultrafine Structure Characterization

Four samples were fully investigated for their inhomogeneity, thickness and density of the collagen based corneal scaffold. The Multiphoton Microscope was utilised with 20 $\times$ objective, with numerical aperture of 0.75, to acquire initial data visualised in three spectral channels of wavelength 433 nm, 520 nm and 667 nm. The scanning steps along the z-axis were set to 0.45 μm and scan speed was set to 166 lines-per-second (lps). The maximal laser power was set to 2 W and resolution pixel in X-Y dimension was set to 0.25 μm^2 . The Auto Quant X3 was used for image deconvolution to facilitate the attenuation correction and 3D blind deconvolution. The thickness of the corneal scaffold was measured by 16 pre-set locations on each Ortho slicer, which is illustrated in Figure

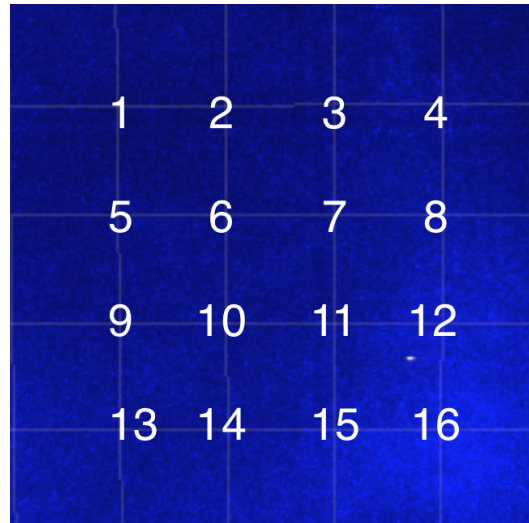


FIGURE 4.20: Illustration of 16 pre-set locations on each Ortho slicer for thickness measurement.

4.20. The sharp edge of the scaffold was identified by adjusting the contrast of the signal and the appearance of signals were noted as the Ortho slicer moves. The measurement points were recorded when the first time signals appeared and disappeared on the Ortho slicer to identify the overall thickness of the corneal scaffold.

The Isosurface module approximates fluorescent signals with the same or higher intensity into the same surface formed by triangular units, thereby excluding noise from empty space, enabling it to accurately identify the interface between dense collagen and void space. The obtained image is used to evaluate the density at each recorded area and reveal the structure inside the scaffold. The identified void space, inside the cubic structure, is assumed to be empty for medium and cellular substrate to occupy. In this section, four samples were investigated where three different locations were measured for each sample, as it is illustrated in Figure 4.21, and the measurement itself was repeated for three times. By comparing Figures 4.21 a) c) e), the density at location two reflects a higher value compared three and one respectively; this shows that the density can vary at different locations on a corneal scaffold, which is further evident from Figure 4.21 b), d) and f). These differences in local density indirectly indicate the porosity on the surface, and may influence medium permeability and potentially limit cell migration inside the

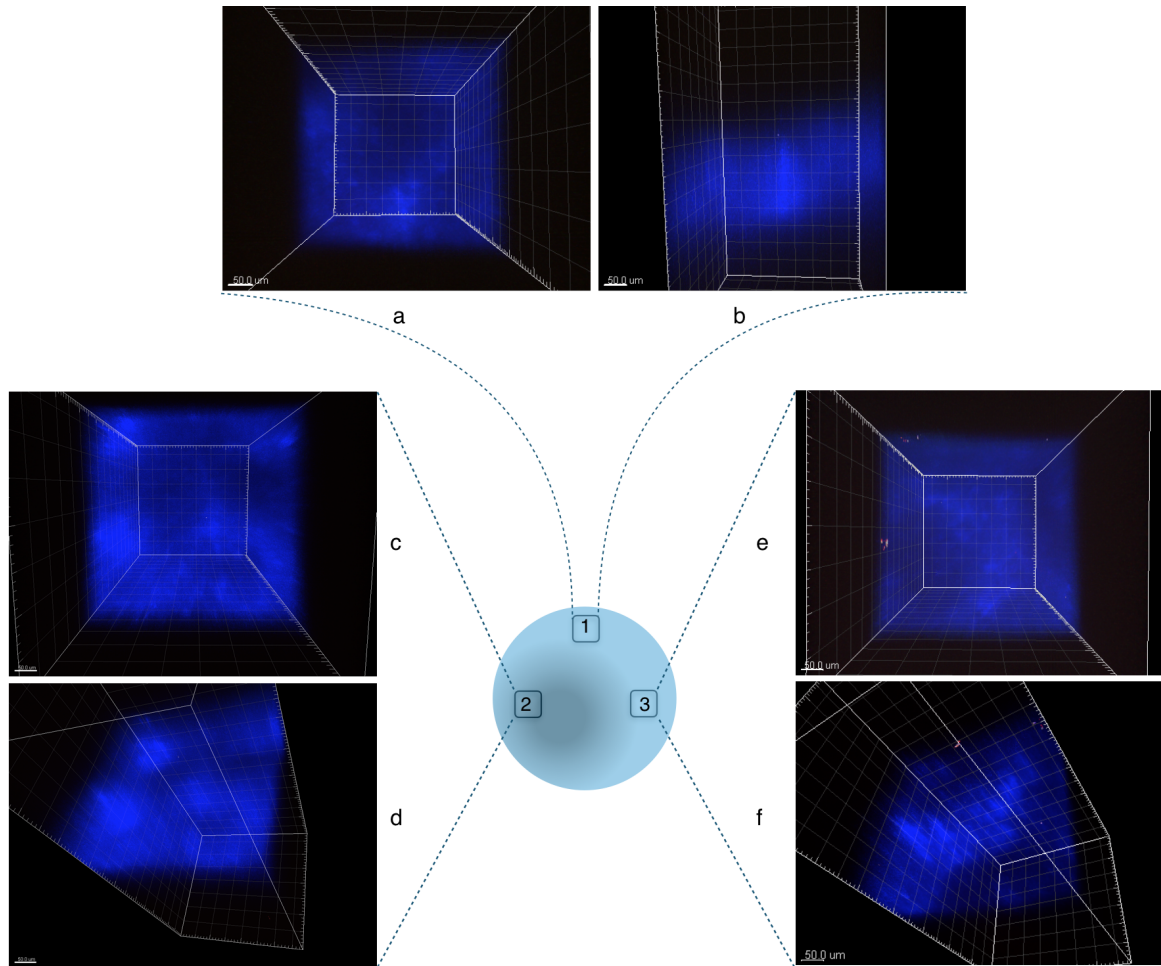


FIGURE 4.21: The MPM images were used to observe how densely collagen forms the structure of scaffold. Different location on a same collagen cornea scaffold showed different local density which may directly influence water permeability or limit cell migration inside the scaffold.

scaffold during the cell seeding process, which will be discussed in next Chapter.

The polyethylene material that surrounded the corneal scaffold did not create visual artefacts for MPM imaging. The thickness of each located area for four corneal scaffold was qualitatively identified during z-axis scanning process and is summarised in Table 4.3. All four corneal thickness lay within the range between $110\ \mu\text{m}$ to $200\ \mu\text{m}$ in dried conditions. The results also revealed that the thickness at different locations on the same corneal scaffold varies from point to point with maximum difference of 23 %. Generally, location one, 3 mm closer to the edge of scaffold compare with other two locations, always shared the smallest thickness among three selected location and

it can be concluded that the thickness in the central area is generally greater than the thickness at the edge. Further analysis and inspection in the dried condition was

	Location 1		Location 2		Location 3	
	Average	SD	Average	SD	Average	SD
Sample 1	163.26 μm	5.47	200.93 μm	3.95	175.23 μm	1.72
Sample 2	134.34 μm	2.67	156.07 μm	1.98	172.86 μm	4.38
Sample 3	119.57 μm	1.93	134.19 μm	4.88	157.26 μm	9.43
Sample 4	169.12 μm	5.83	199.81 μm	4.27	163.79 μm	1.99

TABLE 4.3: Quantitatively identified thickness through z-axis scanning in MPM for four unique collagen scaffold

carried out for other forty-two samples, without identifying the average thickness at three selected locations. Six samples were discovered with a few spots on the edge that were burned during scanning. Burning is defined by both i) spot larger than 2 μm in diameter; ii) abnormal signal shows up on all three RGB channels. One burning example is illustrated in Figure 4.22.

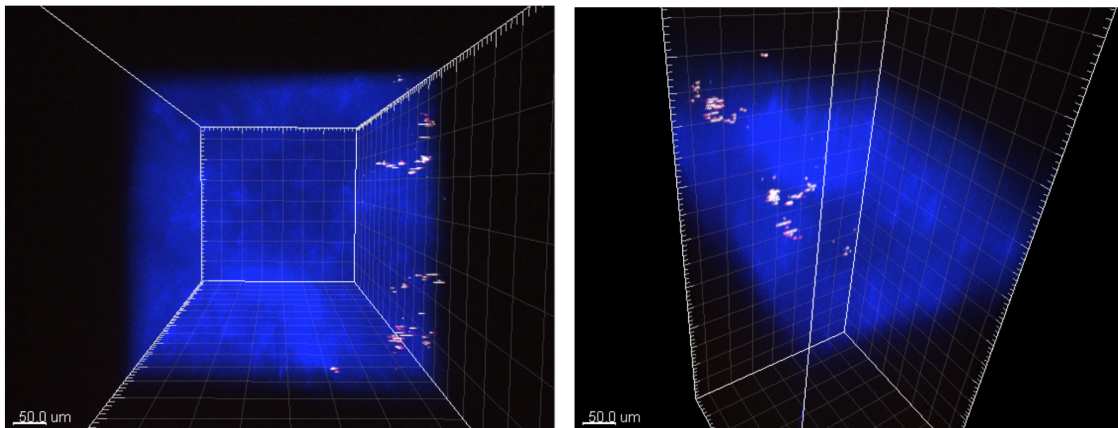


FIGURE 4.22: Burn spots found on the anterior surface of collagen corneal scaffold but does not occur to the collagen compartment

The burning events are only expected to take place when the laser power level is high enough to melt down the inspected material that it encounters. Burning can be reduced by simply using less laser power with high gain factor to compensate for the image

quality. Nevertheless, the majority of the collagen does not experience burning during the imaging scan, indicating that the laser power was not set high enough to burn collagen material. Therefore, the burning event is believed to be caused by some other materials, which could arise from impurities (e.g. cell debris) left from the scaffold processing and tissue treatment.

Furthermore, most burning was only found on the oval surface and anterior side of 3D scaffold and does not occur to the collagen compartment. Since the Bowman's layer contains no cells, it is likely that the burns come from debris of the epithelial cells. The high burning rate from those forty-six samples suggested that the decellularization process might require further optimization.

These six samples with burning spots were excluded from further inspections in the following section. These experiments show that MPM can be utilised as non-invasive ultrafine microstructure detection tool to evaluate and predict the performance of the dried 3D scaffold samples at specific locations before cell seeding intervention. However, the experiments also revealed the thickness on the same corneal scaffold can vary by a maximum 23% in 46 measured samples. The average thickness and average density of each sample was different to each other. Considering the size of the detection area ($500\text{ }\mu\text{m} \times 500\text{ }\mu\text{m}$) and representativeness of each inspected area on the corneal surface, MPM can only be used to provide a general guidance for material's local density and potentially hinted its homogeneity. It lacks the ability to quantitatively summarise the properties of the collagen scaffold over an entire corneal region. Hence the link between Haze parameter and local ultrafine microstructure was not substantially established as the analysed thickness on the corneal scaffold was very dependent on the focus area of MPM.

4.6 Summary

In this chapter, an integrated automated collagen corneal scaffold inspection system was proposed with the attempt to serve as a non-invasive and non-destructive high-throughput screening quality control to identify the crucial optical properties for collagen scaffold. Part of the hypothesis was that, by accurately determining the optical properties of collagen scaffold in dried conditions, it will reveal the biocompatibility between inspected collagen corneal scaffold and seeded cellular substrate.

It was found that the Haze meter, among a few existing inspection technologies, was able to provide an accurate measurement to quantitatively summaries the properties of collagen scaffold over entire corneal region. Due to the requirement for precise overlap between 7 mm aperture and 8-10 mm diameter corneal scaffold sample, the manually measured optical parameter severely fluctuated due to human operational errors. This operational effect was successfully eliminated by designing and integrating an automated inspection system including a XZ motorised mechanical sub-system, a vision recognition sub-system, and an analysing sub-system.

The fabricated system indicated, by employing ANOVA analysis, there is significant difference ($F(2,6)=14$; $p<0.01$) for corneal recognition in a sealed environment under different spectrum and it verified the 590~720 nm to be the most favourable environment for corneal central identification under sealed condition. The absolute displacement error for corneal central position under developed system has been kept within 0.2mm.

Its reliability was tested for 200 hours collectively without any major fault. Furthermore, the optical properties include haze and total transmittance were precisely recorded via the integrated automation system and, by utilizing central limit theorem and 3-sigma rule, were statistically analysed to identifying the optical boundary conditions to assess the quality of corneal scaffold for potential 3D cell culture. Both Shapiro-Wilk and Anderson-Darling tests revealed that the normality of optical properties within selected

bands was significantly drawn from a normally distributed population at 0.01 significant level. The 95% predication bands with narrow interval also indicate a high prescribed probability for the values of future measurements from samples with selected bands.

The ultrafine microstructure investigation was carried out with Multi-Photon Microscope (MPM) followed 3D reconstruction via Imaris software. Despite the fact that MPM can only be used to evaluate and predict the performance of the dried 3D scaffold at specific locations with small focusing area, which is not representative enough for collage scaffold, it can be utilised as an non-invasive microstructure detection tool to provide a general guidance for material purity and homogeneity inside of corneal collagen material but not feasible for high-throughput screening application.

Chapter 5

Real-Time Monitoring of Cell Proliferation on Collagen I Scaffolds

5.1 Introduction

The use of scaffolds to aid in the generation of three dimensional cell cultures is an established tissue engineering strategy, and gaining better understanding cell-scaffold interaction is necessary to advance this approach. Whilst the tissue engineering of endothelial cells and fibroblast cells is relevant for in-vitro investigations that relate to corneal tissue engineering, investigation of other cell types is also of great interest in relation to other culture models and drug testing investigations e.g. to evaluate the efficiency of anti-cancer drugs.

The invasive characteristic of cancer cells is usually assessed in-vitro using Matrigel, which can be viewed as a type of surrogate basement membrane. Cancer cell interaction with type I collagen matrices is also commonly investigated, particularly for study of the peritoneal route to metastasis under-taken by several cancer types, including ovarian cancer [Goodman et al. 2016; Vernon 1999]. Immunohistochemical examinations demonstrate that type I collagen is a major constituent of human corneal stroma [Daxer 1998; Birk 1988]. For this reason, cornea based type I collagen scaffolds can be used as

a generic scaffold for use in investigation of the response of cancer cells and the generalized findings contributes to our knowledge of how cancer cells interact with different biomaterials in microbioreactor.

Furthermore, past literature suggests that most conventional BIS monitoring was associated with 2D rather than 3D cell culture. Different microenvironments were found to greatly impact on cell survival, growth, lineage differentiation and metabolic state [Naderi 2011]. Earlier studies in a simulated 2D microenvironment may be very different to those experienced in clinical trials, and the use of cell therapy applications that have not been thoroughly tested in clinical setting carries significant consequential risks.

5.1.1 Cellular Biology in 2D and 3D Cell Culture

Conventional 2D cell culture techniques, known as a monolayer cell culture, support rapid cell growth and high level production of cellular factors, whereas many commonly used in-vitro 3D cell culture techniques result in slower growth and production rates. However, the speed of cell proliferation and the efficiency of the specific expressed cellular product is often not major concern during the in-vitro culturing process in life science research. From the cell cultures operational perspective, a 2D cell culture is often operated in a well-plate. Seeded cells adhere to the bottom of a tissue culture well-plate. Following the extension of pseudopodia, the cell forms focal adhesion (also known as cell-matrix adhesion [Zamir 2001]) to obtain stable state of anchorage to the bottom of the well [Berrier and Yamada 2007]. Larger fibrillar adhesion (FB) can then be formed where the actin cytoskeleton can extend from these sites [Spuskanyuk et al.2008; Chen et al.2003] to provide the driving force at the cell front; meanwhile, the cytoskeleton microtubule network inside the cell assumes a regulatory function in coordinating rear retraction [Wehrle and Imhof 2003], which ultimately causes the cell to be stable and flat at the bottom of each well [Spuskanyuk 2008]. After FBs form, the cell will either

migrate, differentiate or begin to increase in number through a process of proliferation [Bernhard 2003]. However, as the population of cells increases, the surface of the dish may become crowded. If the the cell population density exceeds a certain amount, cell proliferation rate will decrease; this is known as contact inhibition [Berrier and Yamada 2007]. The level of confluence at which contact inhibition occurs varies from one type of cell to another [Gal 1981]. The level of confluency is similar to the degree of cells coverage of the tissue surface found in the in-vivo microenvironment of the human body, and it often less than 100%. Due to cell-cell interaction, where the monolayer culture surface should never be fully possessed by the cultured cells [Hayflick 1965]. However, in some types of 3D culture such as hydrogel cell cultures, FBs are less likely to occur, and the cell will retain a more spherical shape, adhering to the scaffold and stabilize in space rather than flattening on a 2D surface. In this type of 3D cell culture, less cell contact inhibition will take place and each cell is fully exposed to the scaffold environment. A comparison of 2D and 3D cell adhesion behaviour is shown in Figure 5.1.

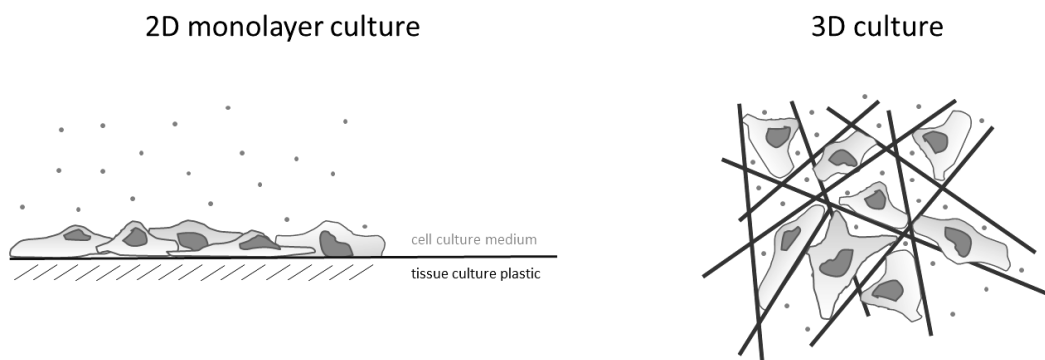


FIGURE 5.1: Illustration of the in-vivo microenvironmental difference for cell adhesion in 2D and 3D [Image from On-Line, refers to Website-List]

The key issue is whether cells are capable of maintaining the same trait after being cultured in an in-vitro environment compared with in-vivo proliferation. Past research shows that, in many cases, cells gradually lose their original traits (e.g. functionalities and cellular response) under 2D cell culture conditions, whereas the 3D cell culture environment can generally provide more accurate conditions that are similar to the

physiological conditions of the human body [Cukierman 2001]. However, different types of cells have different in-vitro microenvironments. Some cells, such as endothelial cells, form an interface between the circulating blood or lymph in the lumen and the rest of the vessel walls [Wang et al. 2007]; these cells are more adapted to the 2D cell culture environment rather than 3D cell culture. Thus, depending on the actual cell's in-vivo environment, the microbioreactor system needs to simulate or provide an in-vitro environment as similar as possible to the in-vivo one, with the most important factor being the interaction between the cell cultures. In order to fully understand the cellular response in the in-vitro environment, it is essential to implement the inspections with 2D and 3D cell cultures.

5.1.2 Existing Bioassay for 3D Cell Culture

Real-time in-situ monitoring in 3D is very challenging as cells are suspended within the scaffold and microscopic observation may be hindered by scaffold itself. Among the existing types of bioassays for 3D cell culture, the TrypanBlue is heavily dependent on the precise human operation, since cells can be easily lost in transferring a suspended cell solution from one container to another.

AlamarBlue is also commonly used and has been reported to be able to quantitatively measure the proliferation over a time-lapse cell culture. The indicator is based on detection of metabolic activity and has been successfully used as an oxidation-reduction indication in cell viability assays for bacteria and mammalian cells. However, to quantitatively identify the absolute cell number and the viability of cells from the measured data, a large number of prerequisite experiments are required in order to build a calibrated standard curves prior to performing the viability assay test. The conventional AlamarBlue bioassay procedure is illustrated in Figure 5.2.

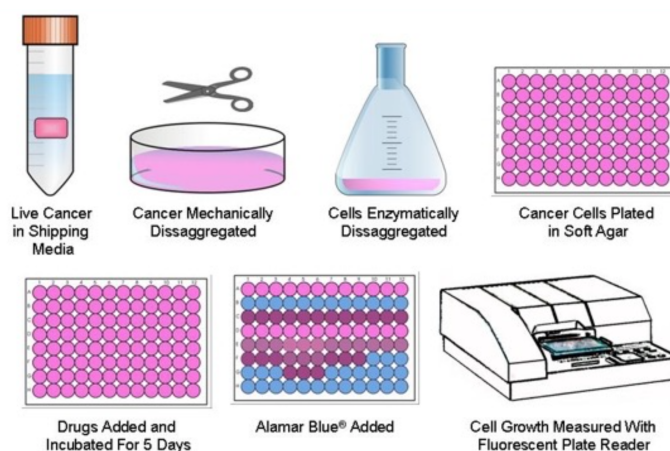


FIGURE 5.2: Illustration of conventional AlamarBlue Bioassay procedure. Image from On-Line, refers to Website-List

Furthermore, the length of incubation time also needs to be determined for each cell type, seeding density and culture environment. The lower the cell density within a culture substrate, the longer the time required for the bioassay to measure cells metabolism. It is particularly difficult to determine the length of incubation time for 3D cell cultures in microbioreactors. If the bioassay is removed too early, it may not have been metabolised enough to be reliably read; on the other hand, the longer the Alamar Blue is in the solution, the more harm it causes to the cells due to change of local pH. Other than the variable time scale required for incubation. A further issue is that the accuracy of results are determined against a control group, which is normally cultured in parallel with the sample groups. Due to human operation errors and sustainability of scaffold materials, the cell number within the control group can become different due to errors in the seeding process and the difference is expected to be largely amplified during proliferation. It is therefore possible that the findings in different control groups run in a parallel experiment may be different to those of the sample groups. Although this issue can be avoided by continuously adding the bioassay into same culture chambers with a regular replacement operation, the scaffold is likely to absorb and maintain the colour from the previous assay and hence constantly bring a non-linear offset to subsequent results.

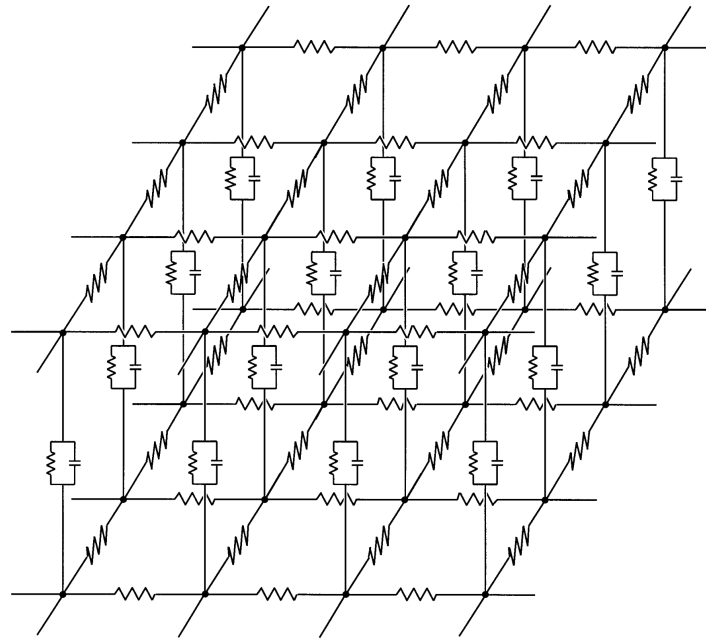


FIGURE 5.3: An equivalent circuit representing a three-dimensional culture environment. Both lower and upper array of resistors represent the extracellular space and scaffold, and the parallel resistors and capacitors represent the suspended cells

Cell growth, cell activity, change in cell composition, shapes and cell location are some examples of processes which can be detected by microelectrode-cell impedance sensors [Muller et al. 2011]. To efficiently manage bioimpedance data, reliable electrical models of the developed system (described in Chapter 3) are required; these include the comprising electrodes, medium and cells. An illustrated electric model for 3D cell culture environment is shown in Figure 5.3. Past literature suggested most studies have only shown the feasibility of using BIS to monitoring tumour cells viability in 2D culture systems, where results shows the BIS magnitude was correlated well with the cell viability [Lei et al. 2012; Yue et al. 2008]. Despite the fact that those aforementioned demonstrations had shown positive outcomes, it is to be noted that those measurement were only implemented in a 2D cell culture environment with bulky BIS machine with undesired physiochemical environment for cellular substrate. Several experiments were carried out in the past three years that use electrochemical methods to monitor 3D cell cultures; these include microfluidic electroporation for cellular analysis and cell growth characterization using multi-electrode BIS [Lu 2013]. Recently, Canail et al. (2015)

developed a system to monitor 3D cell cultures with complementary electrode configurations, where four electrode plates with large surface areas were fully in contact with the cellular substrate. However, the effect of the electrodes impedance was unclear. Nevertheless, as mentioned in Chapter 3, previous BIS monitoring systems were based on existing LCR meters, and only a few attempts were made to quantitatively inspect the 3D cell culture in the microbioreactor platform.

Thus, there is a need to quantitatively monitor the cellular response over time-lapse cell culture on a real-time basis outside of conventional incubation without damaging the cells. In Chapter 3, the concept to utilize miniaturized in-situ BIS measurement for an alternative cellular viability assay in 2D was demonstrated successfully. Hence, the aim of this chapter is to validate the applicability of optically inspected corneal collagen scaffold for cell culture. Meanwhile, the evaluation of feasibility and reliability of μ TAS to quantitatively monitor the cellular response, in comparison with conventional bioassays, was also conducted under the proliferation process.

It should be noted that sensitivity of the fabricated μ TAS system was expected to be rather limited for other cellular response monitoring such as differentiation process. Under such circumstances, the cells are viable but not proliferating. The accumulation of bindings between ligand molecules and receptors activates various complex signalling pathways inside of the cell and the accumulations of activated signalling pathways ultimately activate the transcription factor, which is a protein that can bind to specific DNA sequences [Schaffler 2007; Evans 1998]. It is the different activation sequences for transcription factors that controls the gene expression of the cell, which ultimately defines the differentiation [Reilly 2010]. The above mentioned process either took place on the intracellular area or inside of the nucleus [Olefsky 2001]. The identification of the signal variation within the plasma membrane is beyond the capability of the proposed BIS μ TAS with tetrapolar data acquisition system.

5.1.3 Lung Cancer Cell Investigation

In response to the previously mentioned challenges and needs, the Lung cancer cell was selected to be inspected in both 2D and 3D cell culture, as Lung cancer is listed as one of the most fatal cancers in the world. The malignant changes of the epithelial structure were considered vital steps in lung cancer development. There have been many reports regarding the changes in genetic and microenvironment for lung epithelial malignancy, which were verified by microscopic observation. However, the reports on the electrical properties of these malignant tissues are still limited. The change of bioimpedance can potentially provide clues to the electrical properties of the epithelial structure of the lung tissues, which can be employed to predict the malignancy levels for diagnosis and therapy.

AlamarBlue was selected as the bioassay to identify the cellular response to the corresponding microenvironment; microscopic observation was also implemented to assist the findings. The BIS in-situ monitoring was operated immediately after bioassay analysis to provide more accurate results to indirectly indicate the in-vitro microenvironment in PDMS micro-bioreactor culture chamber.

5.1.4 BIS Measurement of Malignancy Level of Cell cultured In Vitro Tissues

Other than the microenvironment, previous studies demonstrated the type of formation for cell adhesion during proliferation, differentiation, and host-biomaterial interaction is largely dependent on the genetic expressions of the cells. The same type of cell with different gene expression can have different behaviour of deformability. Furthermore, many abnormal cell types exhibit adhesion and proliferation characteristics that are

different to their normal counterparts. To distinguish these behavioural characteristics, a space and time resolved measurement system is needed.

The H1299 cell, known to be a cancerous lung cell, and the version used in this investigation had been genetically modified to remove the RASSF1A gene. This genetically modified H1299 cell is hypothesized to form less durable attachments during the formation of 3D tissue, whereas the wild type of H1299 cells are expected to exhibit stronger adhesion during cell culture. H1299 Lung cancer cells were cultured in both 2D and 3D microenvironments with corneal collagen scaolds as well as with matrigel scaffolds. An initial experiment was carried out to characterize the BIS frequency response of matrigel and type I collagen within the PDMS microbioreactor platform. This was to ensure that both materials did not affect the BIS measurement for cellular substrate where the cells are seeded and suspended inside of the scaffold.

The cyclic voltammetric response for potential interface between the selected electrodes and cellular substrate, which contains the corneal scaffold, was assessed. Twelve corneal collagen scaffold samples, which were statistically categorized into three bands in optical properties, were utilized for biocompatibility comparative studies. The cell proliferation of the seeded cellular substrate (e.g. wild type H1299 cancer cell) was evaluated both by microscopic observation and portable in-situ bio-impedance monitoring for 9 days using the automated ITO heating system. The tendency between estimated total cell number and selecting bands in optical properties was discussed. Moreover, the correlation between quantitatively measured Cole–Cole ratio and scaffold biocompatibility was discussed. The new selection ranges in collagen optical property to indirectly indicate its biocompatibility is re-embedded into the automation control algorithm for future study. In summary, the demonstrated results demonstrate the concept of non-invasive and non-destructive inspection for collagen corneal scaffold for determination of its applicability and biocompatibility with cellular substrate in microbioreactor in cell culture.

Furthermore, the calibration experiment was carried out for cells with and without genetic modification in 2D and 3D culture environment were implemented and evaluated by conventional AlamarBlue assay. The BIS measurement was simultaneously taken along the AlamarBlue assay and the results were confirmed through microscopic observation with cell staining. The calibration experiment was expected to provide a standard calibrate curve between AlamarBlue assay and the corresponding H1299 cell culture metabolic process; it also facilitated the investigation for the correlation between total cell number and relevant BIS measurements. Finally 9 days time-lapse cell cultures on scaffold with both type of H1299 (e.g. genetic wild type and modified cancer cell) were implemented and in-situ BIS measurements were recorded on 3rd, 7th, 9th day respectively. It is hypothesized that BIS will provide a more accurate and non-destructive measurement compared with conventional bioassay methods to quantitatively identify the cellular response (including total cell number and metabolic rate) to its microenvironment in microbioreactor over a time-lapse cell culture. Moreover, it is also part of hypothesis that by measuring BIS, it will reliably indicate the success of gene modification for the selected lung cancer cells. It is to be noted the H1299 cancer cells were provided by CRUK/MRC Oxford Institute of Radiation Oncology.

5.2 Material and Methods

5.2.1 Bioreactor Fabrication

Cyclic Voltammetry analysis followed similar methodologies as described in section 3.2.3 except only platinum was selected as the electrode in the experiments under this section. The fabrication of PDMS microbioreactor followed the similar procedure as in section 2.2.1. Bioreactors were made by the crosslinking of Polydimethylsiloxane (PDMS). The polymers and crosslinking reagent is mixed at the weight ratio of 10:1.05, then the

mixture was poured into an individually designed module. The module was then put into vacuum conditions to remove the bubbles in the mixture. The bubble removal is important to maintain the homogeneity of the inside environment of the bioreactor. After all the visible bubbles were removed, the module with the mixture was then baked 60 °C overnight for solidification. Alternatively, the crosslinking process can be finished in two hours at 70 °C. However, the lower temperature is preferable as some invisible small bubbles in the mixture might take longer time to reach the surface of the bioreactor. Baking at lower temperatures increases the likelihood of removing such small bubbles. Additionally, a PDMS ring with outer diameter of 14 *mm* and inner diameter of 12 *mm* was designed and fabricated. The outer diameter of the PDMS ring shares the almost same dimension with the PDMS culture chambers.

5.2.2 Cell Maintenance

Lung cancer cell line H1299 cells (ATCC, UK) were cultured in high-glucose Dulbecco's Modified Eagle Medium (DMEM, Lonza, UK) supplemented with 10 % (*v/v*) fetal bovine serum (FBS, Life Technologies, US) and 1 % (*v/v*) penicillin (100 U/mL)-streptomycin (100 g/mL) (PAA, US) were cultured in high-glucose Dulbecco's Modified Eagle Medium (DMEM, Lonza, UK) supplemented with 10 % (*v/v*) foetal bovine serum (FBS, Life Technologies, US) and 1 % (*v/v*) penicillin (100 U/mL)-streptomycin (100 μ g/mL) (PAA, US). All the cells were cultured in a humidified incubator (37°C in an atmosphere of 5 % CO_2). Cells were passaged when they reached 80 % confluence. Before cell seeding for spheroid formation, single-cell suspension was made by mild enzymatic dissociation using a trypsin/EDTA solution (PAA, US). Stock culture was transferred every 3-4 days by seeding appropriate numbers of cells of $(0.5-1) \times 10^6$ (depending on cell type and doubling time) into T75 culture flasks. Cells could be frozen in a 30 % fetal bovine serum and 10 % DMSO supplemented DMEM and stored in liquid nitrogen, but

after being defrosted the cells had to be passaged for at least once before being seeded for testing, and this recovery period usually takes 8-12 days.

5.2.3 2D In Vitro Culture

To culture H1299 cells in 2D, each well of the PDMS bioreactors was coated with 70 μL of Matrigel to make the surface hydrophilic for cell adherence, then after 30 minutes of polymerization suspensions of cells with the designated densities were added to each well at the desired density in 600 μL DMEM supplemented with 10 % (v/v) fetal bovine serum (FBS, Life Technologies, US) and 1 % (v/v) penicillin (100 U/mL) - streptomycin (100 $\mu Lg/mL$) (PAA, US).

5.2.4 3D In Vitro Culture

To create a 3D embedded culture of H1299 cells, a dozen collagen corneal scaffold were selected based on the inspected optical properties which mainly differentiated on haze parameter. The chosen corneal scaffold were then taken from the sealed 42 mm petri-dish and were immersed in Phosphate-buffered saline (abbreviated PBS) for 48 hours before seeded into culture chamber. An additional PDMS ring with a diameter of 14 mm was firmly pressed to the bottom of the culture chamber to permanently fix the scaffold position within the microbioreactor chamber. For the corresponding cell density, the total cell numbers of both 2D and 3D in vitro cultures were the same for each well of the bioreactor, to ensure the results are comparable for those two models.

A feasibility study of 9 days time-lapse cell culture in 3D was implemented. It is noted that the Type I Collagen scaffold was selected over the matrigel scaffold with desired density at 8.0×10^5 with both types of genetic modified H1299 cancer cell. This is because the Matrigels structure became loose after 6 days, and was not fit for time-lapse

cell culture purposes. The Type I Collagen scaffold was chosen based on its optical property, later explained in this Chapter. The scaffolds used in this chapter for time-lapse culture were all categorized into the best quality region. An additional PDMS ring with a diameter of 14 *mm* was firmly pressed to the bottom of the culture chamber to permanently fix the scaffold position within the microbioreactor chamber.

5.2.5 Live/Dead Viability Assay

In addition to AlamarBlue assay, cell viability and morphology were also monitored using live/dead viability/cytotoxicity kit (L3224, Invitrogen). The dead cells were stained by ethidium homodimer-1 (EthD-1) with red fluorescence, which is not permeable to living cells. To stain the living cells, the acetomethoxy derivative of calcein (calcein AM) was used. This compound has no fluorescence, but will be transported through cell membranes of living cells, and degraded into calcein, which has green fluorescence. Prior to staining, the nutrition depleted culture medium was removed and each well was rinsed with PBS twice. The culture content in each well was incubated with PBS containing 4 μM calcein AM and EthD-1 in darkness at 37°C for 30 min. Then samples were washed with PBS prior to mount on slides with mounting medium and visualized using an Inverted fluorescence microscope (Nikon, Eclipse TE 200).

5.2.6 AlamarBlue Assay for Cell Metabolic Activity

Cell metabolic activity, or cell viability in each culture well was assessed by AlamarBlue assay (DAL1025, Invitrogen) at each time point by adding 30 μl AlamarBlue reagent solution in each well and incubating for 2.5 hours. After incubation, 50 μl of medium from each well was carefully taken and the fluorescence intensity at 560 *nm* excitation and 590 *nm* emission was measured using a fluorescent micro-plate reader (WALLAC VICTOR2 1420 multilabel counter model, Perkin Elmer, UK). 50 μl of cell-free culture

medium with 5 μ l AlamarBlue reagent solution was set as a blank. The correlation between cell seeding densities and viability, from AlamarBlue, was measured.

5.2.7 BIS Measurement

After the seeding procedure, the microbio reactors were left in incubator for 10 hours to allow the cells to attach and potentially penetrate onto collagen scaffold. The cultured cells were observed under the Nikon Eclipse Ti fluorescence microscope without staining and simultaneously, the bioimpedance spectroscopy inspection was implemented where measuring procedure same as described in Chapter 3 in Material and Methods section. The sweep frequency was set between 1 kHz and 1 MHz with 200 Hz interval and measurement was incremented for 4995 times to obtain the BIS of inspected biomaterial at upper frequency limit. All BIS measurements were taken with 1 V excitation signal from AD5933EVAL combined with tetrapolar data acquisition system in this chapter. Moreover, the electrode is made of platinum as determined in Chapter 3 in electrode characterization section, with a 0.35 mm diameter rod shape with 15 mm length and 4 mm of electrodes length are in contact with culture medium and it is also 0.6 mm above the bottom surface of PDMS microbio reactor chamber.

Data are presented as the mean $\pm$ the standard deviation of the mean of triplicate experiments. For the comparison of total cell number after two days static cell culture, Student's t-test with a statistical significant level of 0.01 was used.

5.3 Results and Discussions

5.3.1 Evaluation of Collagen Scaffolds with Different Optical Properties

Previous chapter demonstrated the capability of integrated system for non-invasive inspection of type I collagen scaffold. By having established three bands for type I collagen scaffold based on its inspected optical property, there is a need to evaluate the impact to time-lapse cell culture from scaffolds with different optical properties.

All culture chamber were seeded with same genetic modified A^+ cell density at 1.4×10^5 cell/mL, the analysis of the images on 3rd day showed that the initial cell density on the corneal scaffold surface was lower in the samples with higher haze parameter which is illustrated in Figure 5.4 (a) and (b) and both images were taken with low magnification using a brightfield setup with Nikon TiE inverted microscope.

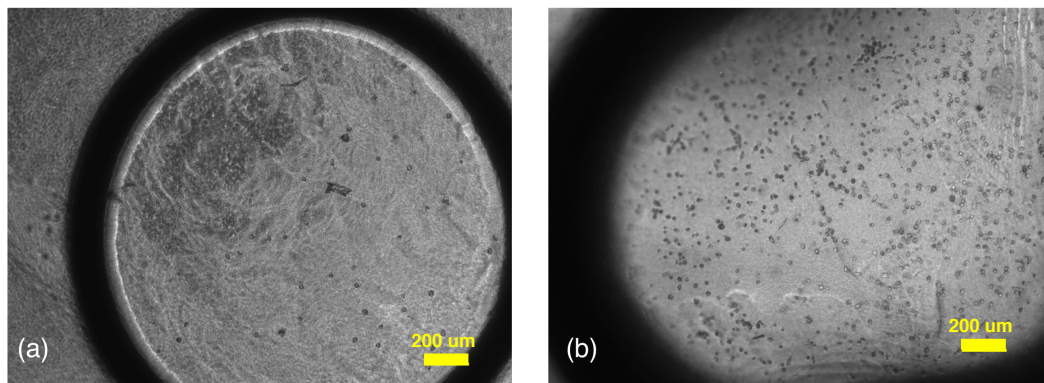


FIGURE 5.4: Bright-field Image of of corneal scaffold sample with A^+ cell seeding density 1.4×10^5 cell/mL on third culture day under Microscopic observation with $4\times$ lens setting. (a) Corneal scaffold with Haze value of 25.18; (b) Corneal scaffold with Haze value of 9.86

As discussed, the optical haze parameter provides an indication of collagen surface roughness and internal scattering structure. The higher the haze, the more likely the surface is

predicated to be rough for cell adhesion and the unevenly distributed density inside the collagen material enables some particles to act as 'scatterers' that cause diffuse scattering. The assumption was made that neither surface roughness nor unevenly distributed density inside of collagen material will facilitate the suitability of microenvironment for cell culture and as a result making cell adhesion difficult to take place.

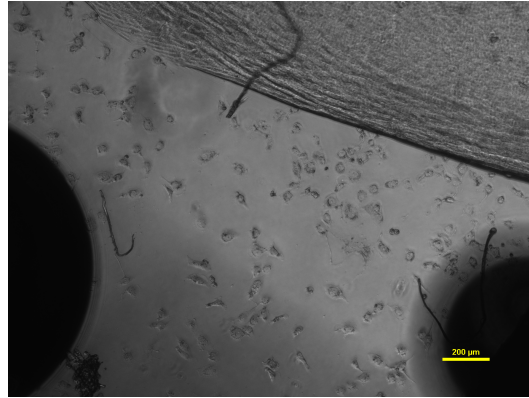


FIGURE 5.5: Bright-field Image on the edge of corneal scaffold (Haze value inspected to be 25.18) on day 3 under Microscopic observation with 4× lense setting

Interestingly, three days after static cell culture, the cell density on the surface of corneal scaffold with haze value of 25.18 remains unchanged to its initial seeding density ($1.4 \times 10^5 \text{ cell/mL}$). In contrast, the cell density increased drastically compared to its initial status. Further microscopic observation for corneal scaffold with haze value of 25.18 proves that most of seeded cells migrated from the rough collagen surface to edge underneath the PDMS ring and form an adhesive cluster on the bottom surface of PDMS culture chamber, which is shown in Figure 5.5. In contrast, the cell adhesion and proliferation for corneal scaffold within the haze parameter 9.8 were continuously investigated for 9 days under microscopic observation using 20× lens (illustrated in Figure 5.6). The continuous data recording proves that cells can be adhesive onto the surface of corneal collagen scaffold and proliferated with desired growth factor in a static culture environment and thus become immobilised. In Figure 5.6 (c) it appears some of the cells began to stretch themselves to be flattered on the corneal surface, whereas a

few cells were trying to penetrate to the inside of collagen scaffold to form a 3D-esque environment.

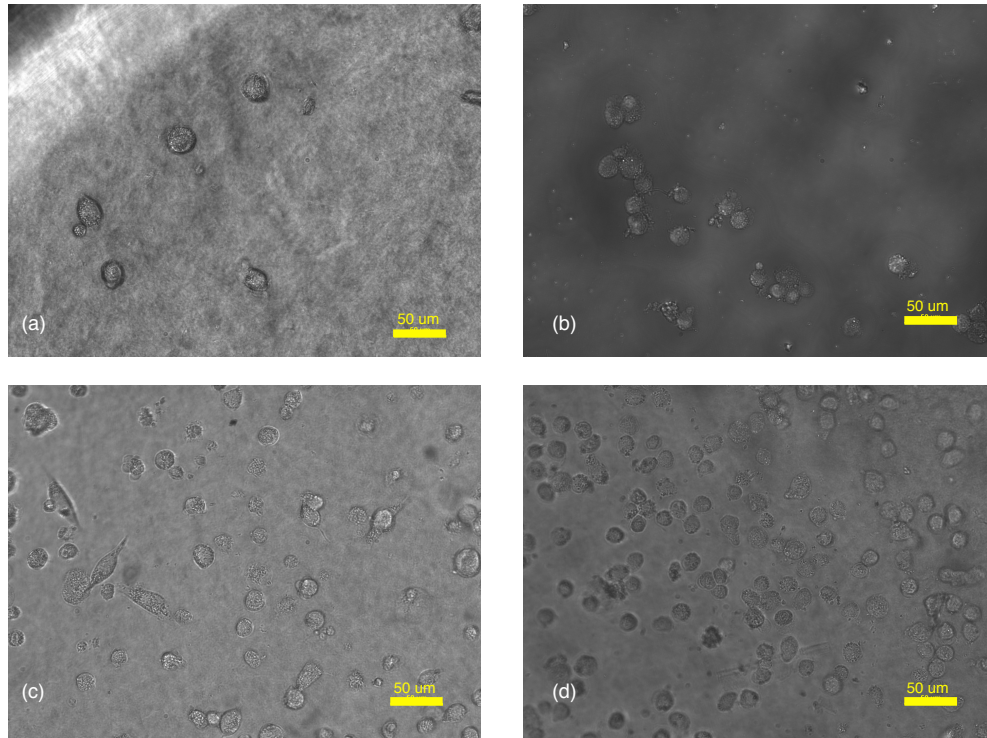


FIGURE 5.6: Bright-field Image of cell culture seeded onto corneal scaffold (9.8 Haze value) with of $20\times$ microscopic lens setting (a) 1st day culture; (b) 3rd day culture; (c) 7th day culture; (d) 9th day culture

The fluctuating characteristics of the collagen scaffold surface was certified by implementing a number of multi-layered image scanning for cellular substrate inside of PDMS culture chamber on day 9 with a depth of $44\text{ }\mu\text{m}$, and is illustrated in Figure 5.7. As the focus changes along the vertical axis, the different cells clusters, which are all firmly attached to the scaffold surface, are exposed at corresponding levels under microscopic observation. The obtained image not only certified the fluctuation of scaffold surface but may reveal the majority of the cells that choose to adhere to the collagen surface and form a 2D monolayer-liked cell culture environment, rather than penetrate the inside of the scaffold to form a real suspended 3D microenvironment.

For cell attachment and migration, the surface of the etched polycarbonate films needs to be densely coated with an extracellular matrix (ECM) protein such as collagen or

laminin that has peptide binding sequences for integrin binding [Zaidel-Bar 2004]. Cells tend to migrate up gradients of ECM from lower density integrin binding sites to higher density, as they are able to build stronger focal adhesion (FA) plaques on denser integrin binding sites [Chen 2003]. This allows for larger bundles of actin cytoskeleton filaments to grow from the plaques, leading to more pulling towards the richer ECM side of the cell.

Ideally, the ECM density is evenly distributed within scaffold that permits an ideal degree of integrin binding so that FA plaques can rapidly form ahead of the cell and dissolve at the rear of the cell and then cells will explore the local surface [Giancotti 1999]. In such a case, pore size, scaffold structure, diffusion of factors from inside the scaffold, cell seeding density, quality of cells and culture medium are important factors that can affect cell migration into the compartment of the scaffold. The surface of a scaffold is considered to be flat and presents a continuous surface for dense focal adhesion formation, whereas the scaffold interior can be densely fibrous and difficult to form dense FA plaques [Harley 2009]. Cells are required to be healthy to express sufficient matrix metalloproteinase (MMP) enzymes to break down and migrate through collagen scaffolds, slowing down migration [Snoek-van Beurden 2005]. A genetic modification of the H1299 cell may cause cells to fail to meet such criteria.

Furthermore, the firm network structure of the pores assists in guiding and promoting new tissue formation [Chevalier 2008; Sung 2004]. The collagen scaffold was discovered to have small porosity (5-10 μm) and cells can generally migrate through pores as small as 8 μm in diameter. This is the size usually used in migration assays where cells migrate through track etched polycarbonate films. Literature suggested it can even squeeze through 3 μm pores by deforming their nucleus [Murphy 2010; Hollister 2005]. However, the small porosity slows effective release of biofactors such as proteins, genes, or cells and may not provide sufficient nutrient exchange for cellular substrate.

The ECM protein was assumed to be evenly distributed with adequate density, whereas the collagen scaffold, as discussed in Section 4.5, was proved to have high but non-uniform local density and it is lack of uniform spatial distribution of medium within them. As a result, the high integrin density may prevent the movement of actin cytoskeleton filaments and FA plaques became too dense to disassemble [Manzanares 2009; Huttenlocher 1997].

The cell density at $1.4 \times 10^5 \text{ cell/mL}$ may be too low for cell to diffuse into the scaffold. Past studies revealed, at lower seeding density, that cells would sit still on the scaffold surface. In contrast, higher seeding density means that the dense cells on the surface express more of their own growth factors, ECM and enzymes into the scaffold, making it easier for cells positioned above the pores to explore into the scaffold [Mandal 2009].

Additionally, collagen scaffold has relatively weak mechanical strength due to its cross-linked polymeric network, making it difficult to maintain its structural stability during the cell migration process [Qiu 2013; Haugh et al 2011]. The thickness of $450 \mu\text{m}$ - $500 \mu\text{m}$ for collagen scaffold could also affect the cell migration since cell density slows diffusion into the scaffold from the top surface. A thin scaffold with firm structure will allow cells migrate away from the cell mass and toward factors diffusing through the scaffold.

Although a true 3D microenvironment was not formed, nevertheless microscopic observations revealed a well-established cell-scaffold interaction, which ultimately facilitated our understanding of cell behaviour and its compatibility with the engineered scaffold. Figure 5.8 illustrates the microscopic observation for 9 days static cell culture with 12 corneal collagen scaffolds, which were selected from 3 different ranges named 9 - 12, 17 - 19 and 22 - 26 respectively. The estimated cell number was counted based on the microscopic observation and each viability value was obtained by averaging the counting number on 5 separate images captured in bright-field.

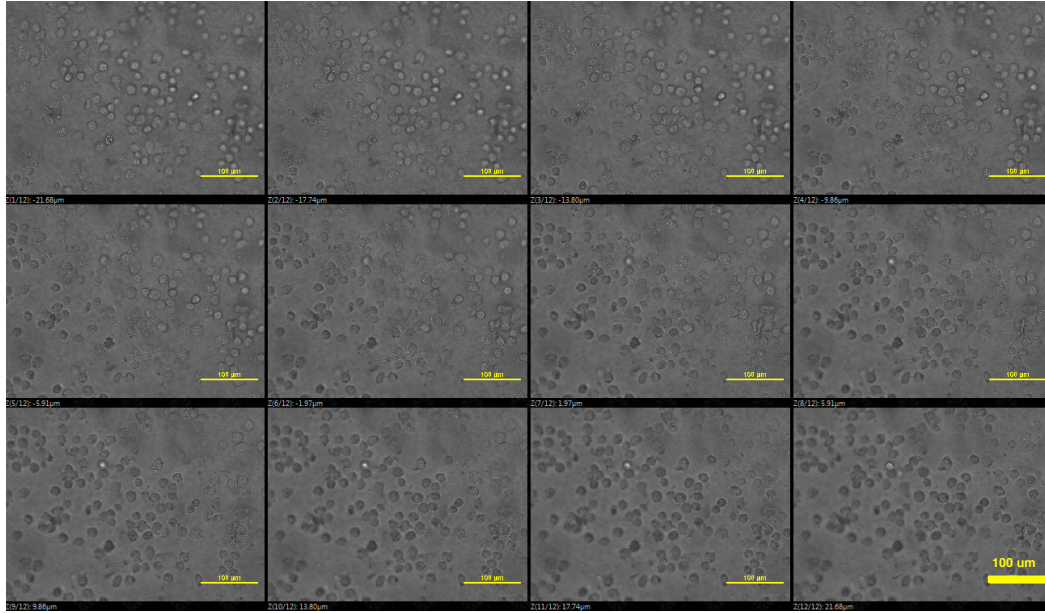


FIGURE 5.7: Multi-layered bright-field Image of A^+ cell culture seeded onto corneal scaffold (which was inspected with 9.8 Haze value) on day 9 with scanning depth of $44 \mu m$ under of $10\times$ microscopic lens setting

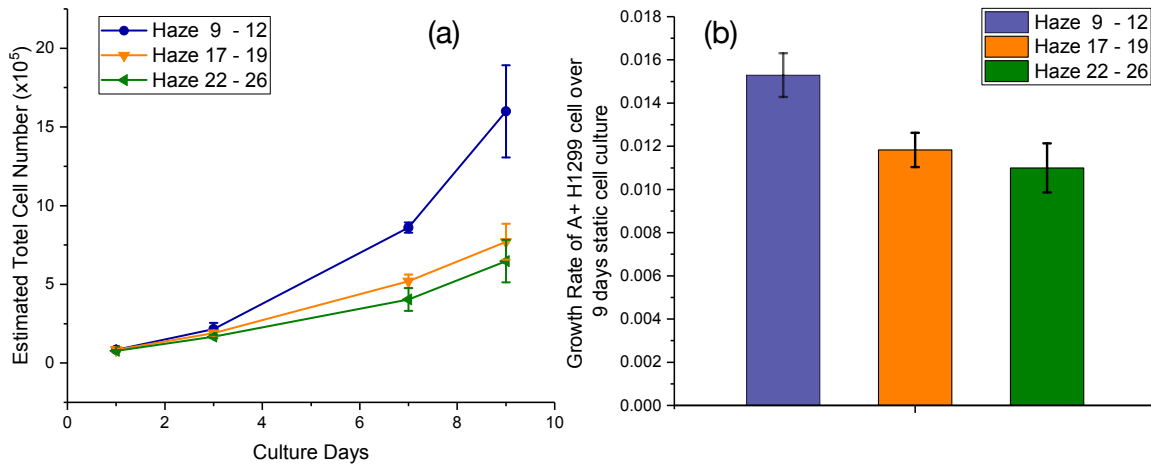


FIGURE 5.8: (a) Total cell number for H1299 A^+ cells in corneal collagen type I scaffold with different Haze parameter estimated by averaged microscopic observations. For each Haze range 3 scaffolds were embedded into culture chamber. For each chamber with designated Haze valued scaffold, at least 5 fields of view were imaged and number of cells averaged and extrapolates against the visible area to obtain a total number of cells per well; (b) Growth rate of cell culture in designated scaffold with different Haze parameter

From Figure 5.8, the best growth rate for cell culture with engineered scaffold was proved to be corneal collagen scaffold with the smallest haze parameter range. In such range, the estimated average for total number of cells had increased from 0.84 ± 0.11 to $15.96 \pm 2.93 \times 10^5$ cells whereas the growth factor for cell culture seeded into higher haze valued corneal is lower by more than 50 % comparatively. The general effect introduced by the different haze valued corneal scaffold showed a clear tendency that is as the haze value increases, the growth rate decreases accordingly. This is mainly caused by the fact that higher value of haze indicates a rougher surface on the scaffold and less uniform density/thickness across the collagen which, by observation, forces cells to migrate or fall off the edge of the scaffold. Although the formation of cellular clusters on the edge were observed, the very limited proliferation space and very low surface-to-volume ratio makes it impossible to sustain a large number of newly cultured cells, hence hindering the overall proliferation.

Furthermore the calculated growth rate over 9 days of the static 3D cell culture demonstrated a significant difference ($p = 0.005 < 0.01$), with level of $15.29 \pm 1.01 (\times 10^{-3} h^{-1})$ and $11.83 \pm 0.79 (\times 10^{-3} h^{-1})$ for H1299 cell culture with different scaffolds, where their haze value belongs to 9~12 and 17~19 group respectively. As a whole, this evaluation illustrated that the growth rate of the cultured A^+ H1299 cells with engineered scaffold is highly affected by its optical property where its Haze parameter can be pre-determined non-invasively via optical inspection system in a dried and fully sealed condition.

5.3.2 Cyclic Voltammetry Analysis for Corneal Scaffold

The cyclic voltammetric response was first implemented with platinum electrodes where the electrodes were inserted into DEME medium in PDMS culture chamber and the surface of electrode was set to be 0.5 mm distant away from the corneal scaffold surface without touching each other.

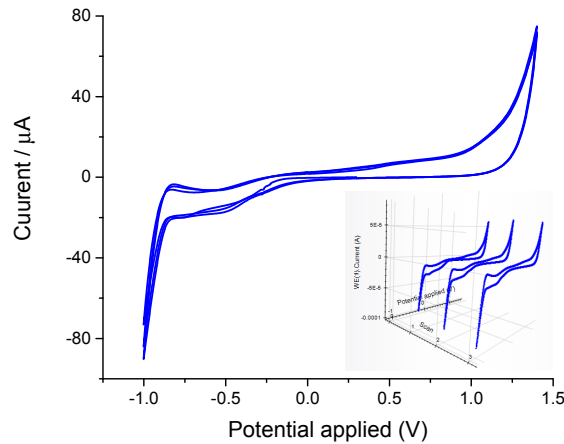


FIGURE 5.9: Cyclic Voltammetry for Pt electrode in medium solution with collagen scaffold without adhesive cells

The Figure 5.9 represents the voltammetric response for Pt electrode in culture chamber where no H1299 cells were suspended on the surface of corneal scaffold. An excellent consistency, in terms of shape of scan as well as the magnitude for repeated operation, was observed during the multi-scan and the capacitive current remains to be small. A similar set of experiments were implemented except the A^+ H1299 cell, which were pre-seeded onto corneal scaffold and already incubated at 37 °C for 2 days. The results are illustrated in Figure 5.10, once again, an excellent repeatability of electrodes CV response was observed.

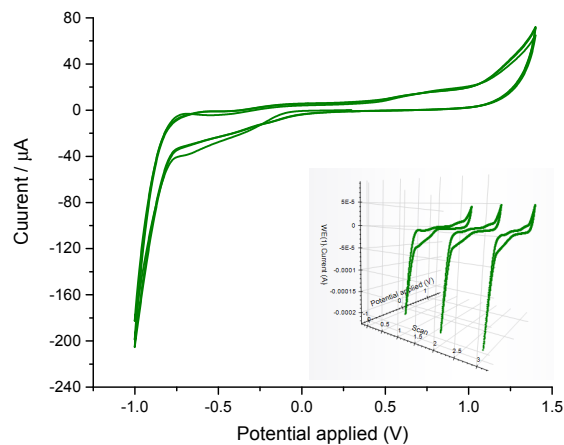


FIGURE 5.10: Cyclic Voltammetry for Pt electrode in medium with collagen scaffold and fully suspended H1299 A^+ cancer cells

Interestingly, the capacitive current was reduced as the cell seeded into the scaffold

as illustrated in Figure 5.11. It is to be concluded that the previously selected platinum electrode does not form an interface with cellular substrate where a collagen based corneal scaffold is added to the culture chamber and hence does not introduce the electrode effect to the Bio-impedance spectroscopy measurement.

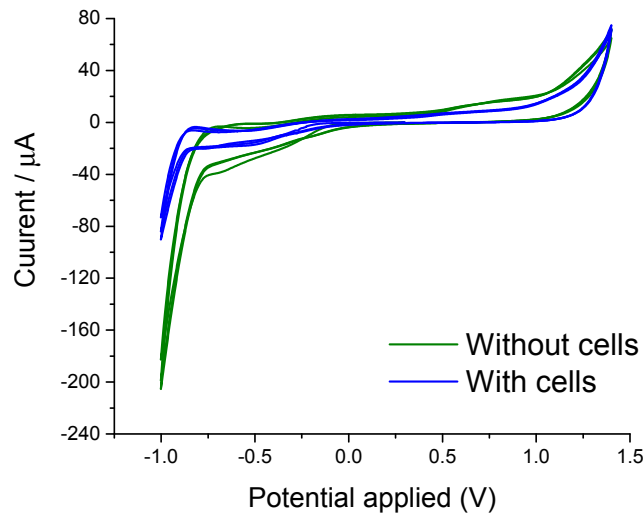


FIGURE 5.11: Comparative Cyclic Voltammetry response for Pt electrodes with and without adhesive cancer cells in medium

5.3.3 Calibration Between Functional Assays and Cell Density

5.3.3.1 Live/Dead Viability Assay

It is noted that the microscopic observation was carried out with cell staining after the BIS measurements took place not only to confirm the seeded cell density, but also the to illustrate the limitation and drawback of conventional bioassays.

The indication for cell density at each culture chamber is clearly illustrated. However, it is impossible to quantitatively compare and conclude the metabolic status or even total

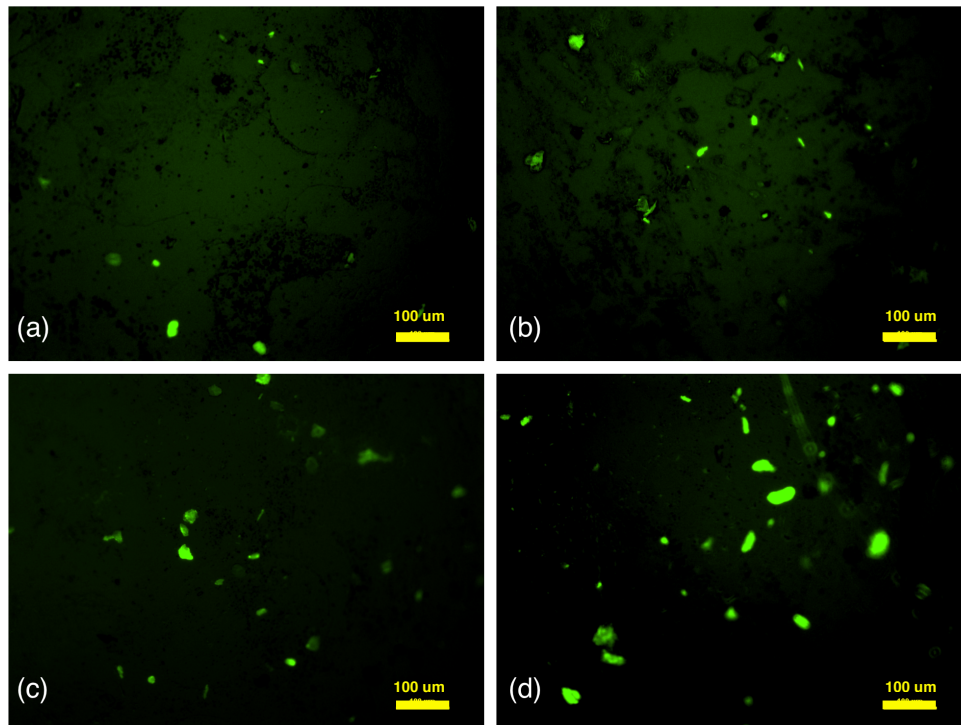


FIGURE 5.12: Live/Dead Viability Assay for C^+ H1299 Cells in 2D with different cell density : (a) 0.625×10^5 ; (b) 1.25×10^5 ; (c) 2.5×10^5 ; (d) 5×10^5 cells/ml

cell numbers between A^+ , which is illustrated in Figure 5.13, and C^+ on the same density level.

The complication of microscopic observation increases as culture environment moves from 2D to 3D. The Figure 5.12 (c) and (d) demonstrated the microscopic image for C^+ in 3D cell culture environment with 2.5×10^5 and 5×10^5 density respectively. The accuracy and reliability of observation cannot be guaranteed as it largely depends on the field of view and interval on the Z-axis during multi-layered scan. Based on the image, it can only be concluded that the maximum distance for A^+ cells distributed along vertical axis is $216 \mu m$ for cell density with 5×10^5 whereas the maximum distance on Z-axis for C^+ with doubled cell density is $789 \mu m$. The conventional staining methods such as live/dead cytotoxicity kit do provide a general guidance and the estimation of cellular activities in 3D environment can also be improved by multi-layered scanning along vertical-axis of culture chamber, but it is limited in providing a quantitative viability and proliferation analysis.

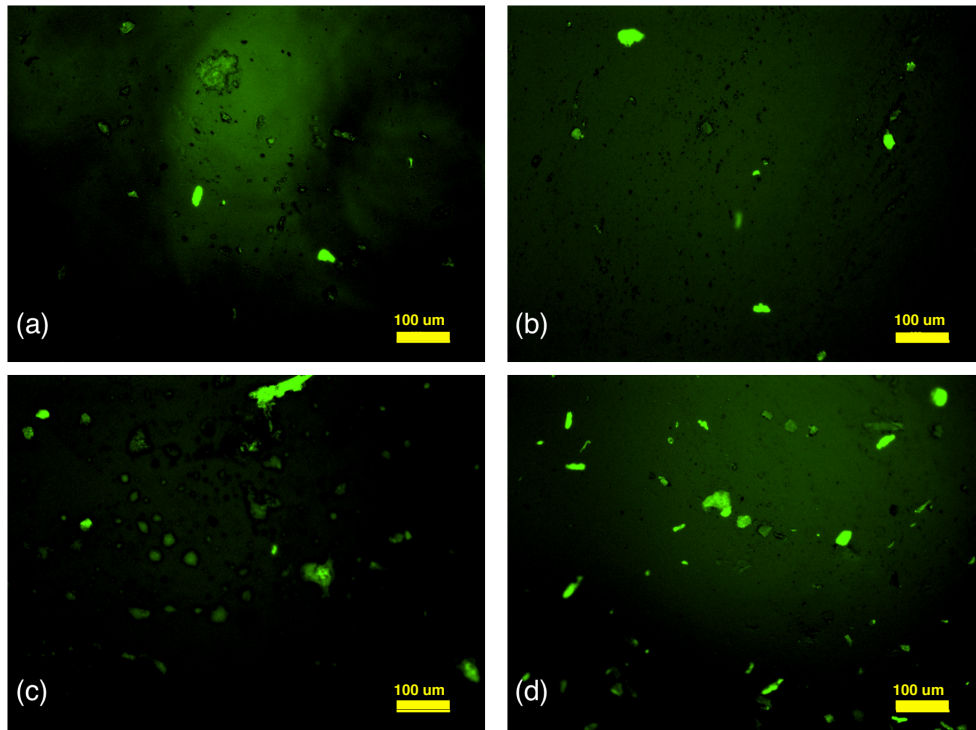


FIGURE 5.13: Live/Dead Viability Assay for A^+ H1299 Cells in 2D with different cell density : (a) 0.625×10^5 ; (b) 1.25×10^5 ; (c) 2.5×10^5 ; (d) 5×10^5 cells/ml

5.3.3.2 AlamarBlue Bioassay

In order to find the more accurate correlation between the seeded cell density with the AlamarBlue bioassay and the corresponding BIS measurement, it is decided to use corneal collagen scaffold in 3D and $70\mu l$ polarized Matrigel in 2D as both setup avoid direct contact with potential measuring electrodes, thereby reduces interference and providing more accurate information.

In 3D cell culture, the H1299 type A^+ (genetically modified) and type C^+ (wild type genetics) were seeded with cell density of 0.625, 1.25, 2.5, and 5 ($\times 10^5$ cells/ml) respectively onto corneal collagen scaffold in each microbioreactor chamber. Additionally, in 2D cell culture, $70\mu l$ of matrigel was added into the corresponding chamber followed with 2 hours polymerization. The microbioreactors were incubated for 24 hours to allow the cell to adhere to its microenvironment. $30\mu l$ of AlamarBlue was later added to each chamber to stain the cells, and density experiments were repeated for three times. The

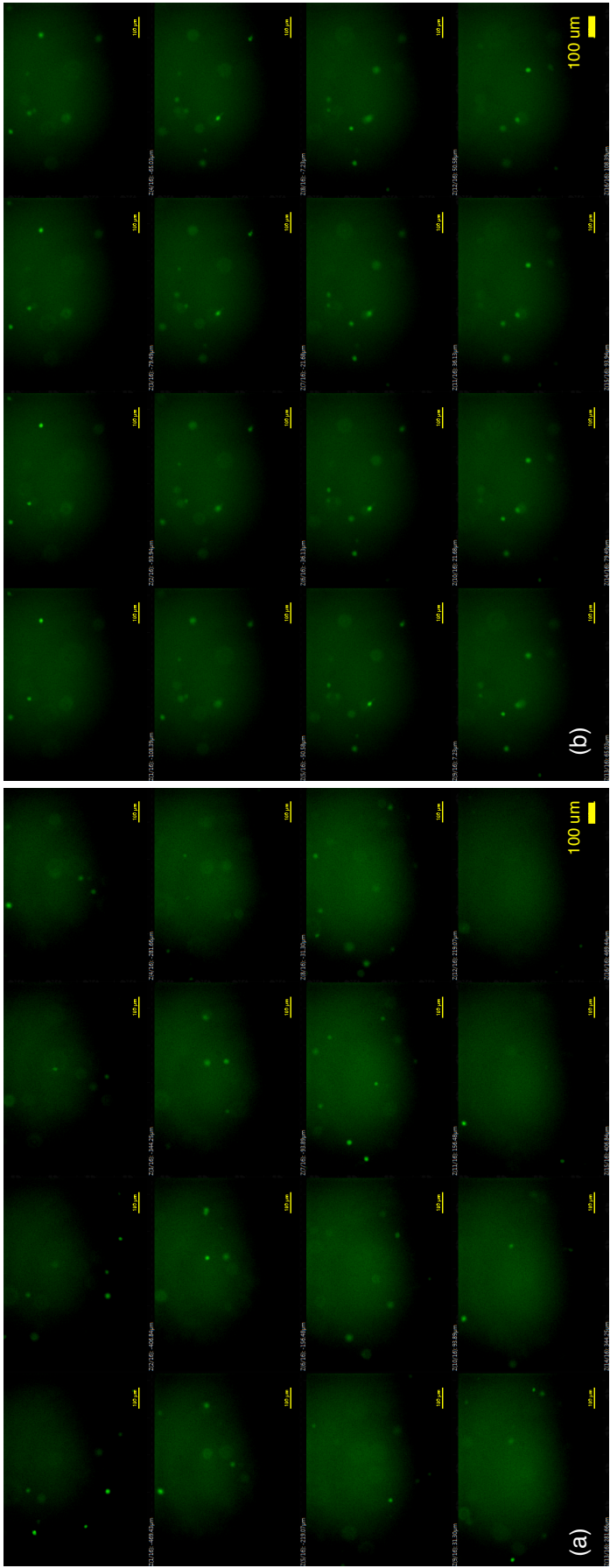


FIGURE 5.14: Live/Dead Viability Assay for two types of H1299 Cells with same cell density of 2.5×10^5 Cells/ml : (a) C^+ cell sample scanned along vertical axis from -469.43 um to $+469.43 \text{ um}$; (b) A^+ cell sample scanned along vertical axis from -108.39 um to $+108.39 \text{ um}$

mathematical expression for AlamarBlue bioassay is shown as following:

$$AlamarBlue_{EnergyAbsorption} \propto \int \sum_{n=1}^N [Cell]_n \times [Viability]_n \quad (5.1)$$

Where the n represent the n th count in cells, N represents the maximum total cell number, $[Cell]$ is an integer which can either be 1 (contain this cell) or 0 (not contain this specific cell). $[Viability]$ represents the specific cells viability and is a decimal number lies between 1 and 0. The integration of accumulated product terms is directly proportional to the absorbed energy in AlamarBlue bioassay measurement and reflects the metabolic process in the culture chamber. This equation will help to understand and build the linkage between cellular biology and relevant inspection measurement. The Figure 5.15 illustrated H1299 cancer cells AlamarBlue measurement with corresponding cell density.

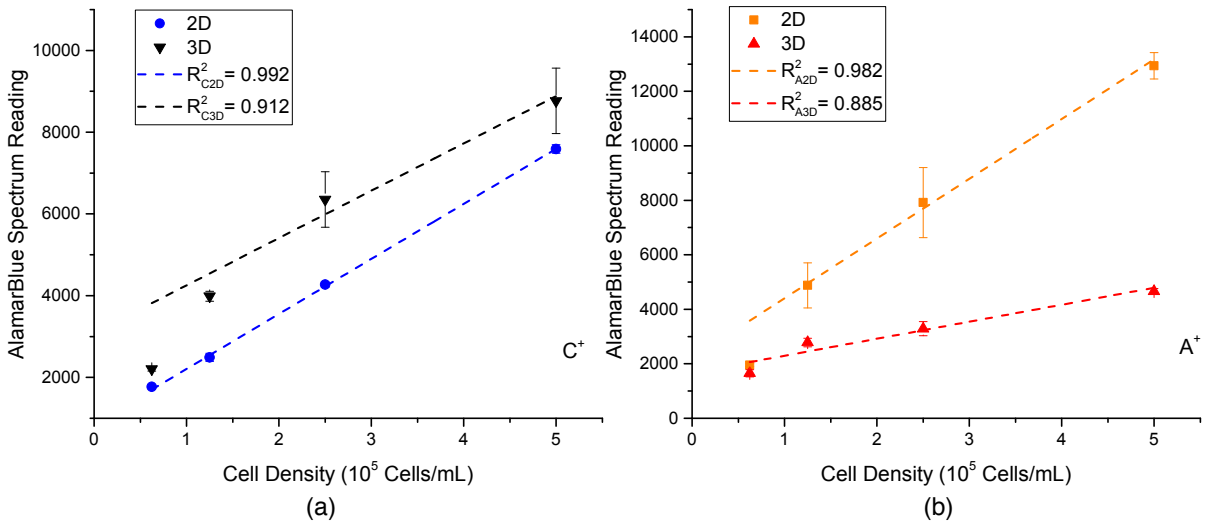


FIGURE 5.15: AlamarBlue Calibration Curve in 2D and 3D culture (a) C^+ ; (b) A^+

From the Figure 5.15 (a) it is clear that both 2D and 3D reflect a good linearity, though the fit for C^+ cell in 3D ($R^2_{C3D} = 0.912$) is not as linear as it is in 2D ($R^2_{C2D} = 0.992$). The general tendency of AlamarBlue Spectrum reading for C^+ in both 2D and 3D does not reflect a significant difference where the seeded cell density lay between $0.625 \sim 5.0 (\times 10^5 \text{ cells/ml})$. The absolute standard deviation error of AlamarBlue spectrum

reading for C^+ with seeding density of $5 \times 10^5 \text{ Cells/mL}$ in 3D cell culture is (± 722) over repeated experiments and is larger than the case in 2D (± 251) , indicating the inadequate repeatability of obtaining metabolic information in a 3D environment. This is partially caused by the fact that some AlamarBlue assays were more absorbed by matrigel and corneal collagen scaffold and the level of absorbance seems arbitrary in different culture chamber, hence it created an unpredictable offset in each assay-based examination.

In contrast, the genetic modified cell, namely A^+ , revealed the metabolic process is very slow particularly in 3D culture format as it illustrated in Figure 5.15 (b). This is due to the absence of gene RASSF1A, it is less able to form durable attachments with corneal collagen scaffold during the suspension.

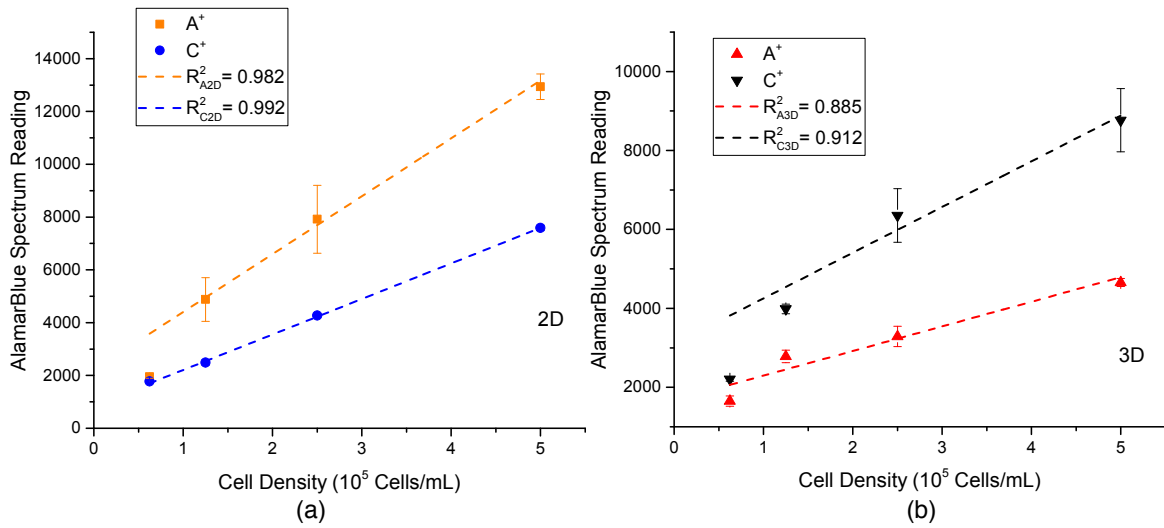


FIGURE 5.16: AlamarBlue Calibration Curve for A^+ and C^+ type of cells in (a) 2D; (b) 3D environment with Matri-gel

Comparing the AlamarBlue test results in 2D and 3D separately, which is illustrated in Figure 5.16 (a)(b), it is found that A^+ is better metabolized in 2D within the range of seeded cell densities with large error ($\delta_{A2D} = 5.3\%$), whereas the C^+ is certainly less metabolized in 2D condition and its performance over repeated experiments is firm. Metabolic performance seems to be swept in 3D cell culture environment, however

the C^+ is more comfortable with microenvironment compared to A^+ and the linearity ($R_{C3D}^2 = 0.912$) fluctuated over repeated experiments in high cell density region, whereas genetically modified A^+ clearly reduced its metabolic process whilst successfully while maintaining a reasonable linear correlation ($R_{A3D}^2 = 0.885$) with respect to cell density.

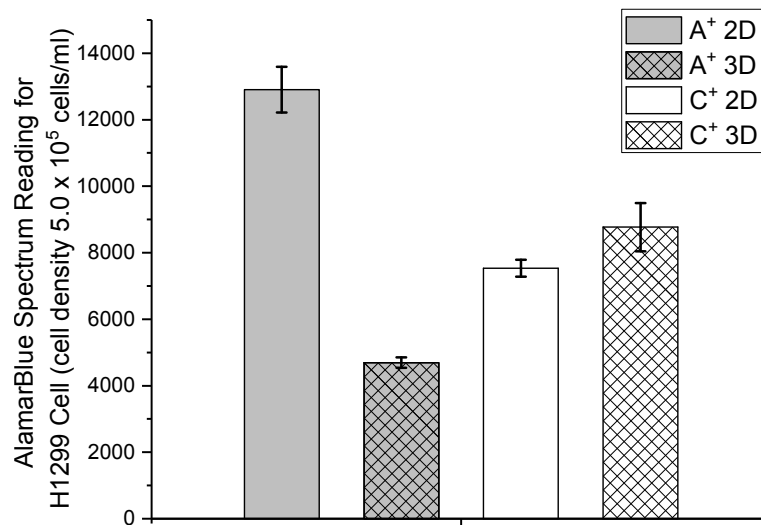


FIGURE 5.17: AlarmaBlue Spectrum Reading for both A^+ and C^+ H1299 cell with designated cell density of 5.0×10^5 (cells/ml)

Figure 5.17 indicates that the reduction of metabolic process of genetically modified cancer cell was clearly observed by employing AlamarBlue bioassay especially in 3D cell culture environment. By adapting A^+ cells in 3D, the metabolic activities reduced by 64% whereas results exhibits a 14.4% increases for C^+ cells by imposing similar environmental transformation. The T-tests with the significance level of 0.01 also revealed the changes of culture environment is significant for A^+ cell culture where $p = 2.0 \times 10^{-5}$ ($p < 0.01$) and statistical analysis for C^+ gives the T-test value of 0.126 ($p > 0.01$) and thus C^+ metabolic activities would not change significantly if the culturing environment

is changed. The percentage errors ($\delta_{C3D} = 9.3\%$) of using AlamarBlue assay for indication of metabolic status in 3D cell culture are larger than percentage errors in 2D ($\delta_{C2D} = 3.3\%$) and hinders further 3D analysis. It is therefore concluded that staining methods can offer a general guidance but lack quantitative determination.

5.3.3.3 BIS Characterization for Scaffold

Matrigel is a common scaffold in 3D cell culture. To characterize the Matrigel scaffold prior to monitoring of 3D cell culture, each 100 μl , 200 μl and 250 μl matrigels were added into three separate PDMS culture chambers, with pre-embedded Pt electrodes in the microbioreactor system. The polymerization was implemented immediately after the matrigel was introduced into chamber and was incubated for 30 minutes. Culture medium of 250 μl was carefully added into the culture chamber and maintaining its position on-top of polymerized matrigel. BIS measurement was implemented from 1 kHz till 100 kHz and the results are illustrated in Figure 5.18.

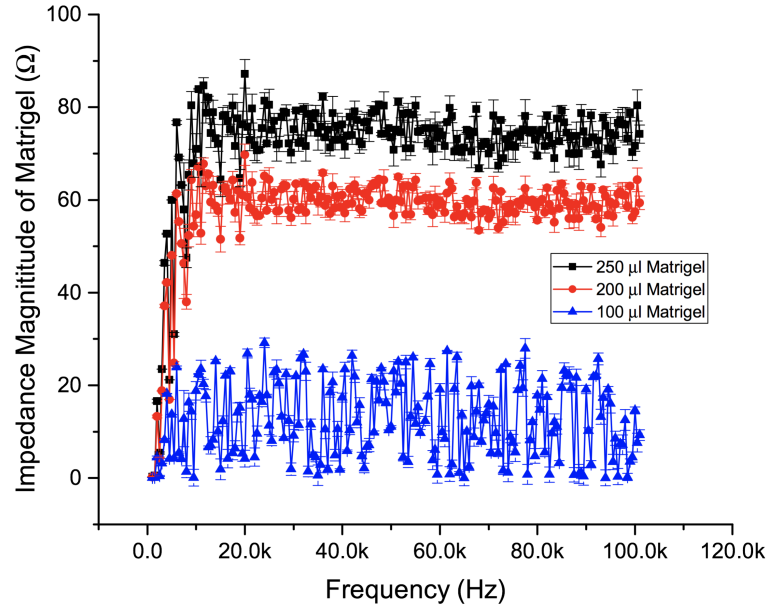


FIGURE 5.18: Measured bioimpedance for Matrigel scaffold with different different sizes after polymerization

The accumulation of charges produce distinct dielectric properties on both sides of membrane is the is the major factor that causes variation of bioimpedance over low frequencies range. From 5.18 it can be seen that bioimpedance magnitude of different volume matrigel in the bandpass region does not change as the frequency of the exciting current increases. Thus, the substrate such as matrigel and culture medium can be considered as an ionic conductor and from electrical perspective, it contributes a constant positive offset in BIS monitoring for 3D cell culture, provided the given amount of matrigel remains unchanged.

Moreover, it can also be seen that the magnitude of bioimpedance for matrigel with different quantity generally decreases as the quantity of scaffold decreases, but not strictly in a linear fashion. Considering the dimensions of the chamber, the bottom surface area is 186.26 mm^2 . Hence polymerized $100 \text{ }\mu\text{l}$ matrigel would only contain the depth of 0.54 mm and is smaller than 0.6 mm which was set to be the distance between electrodes and bottom surface PDMS culture chamber. Based on the tetrapolar measurement principle, the measured bioimpedance directly reflects the proximal or constructional cellular substrate zone along the equipotential surface that lies between two potential electrodes. Hence, there is a need to classify the BIS measurement in 3D cell culture, as direct contact between potential electrodes and the matrigel would result in different levelled offsets. The threshold volume on this proposed microbioreactor platform is $112 \text{ }\mu\text{l}$ of matrigel and therefore in 2D cell culture, BIS value is considered to be significantly small compared with BIS value of cells.

The characterization of Type I Collagen Scaffold followed similar procedures compared with matrigel and the results are illustrated in Figure 5.19. Despite the stabilized bioimpedance of the material over a large frequency spectrum, the magnitude of collagen scaffold is much smaller in comparison to matrigel gel. This is due to the fact that, after treatment in PBS solution for continuously 48 hours, collagen scaffold is expended to

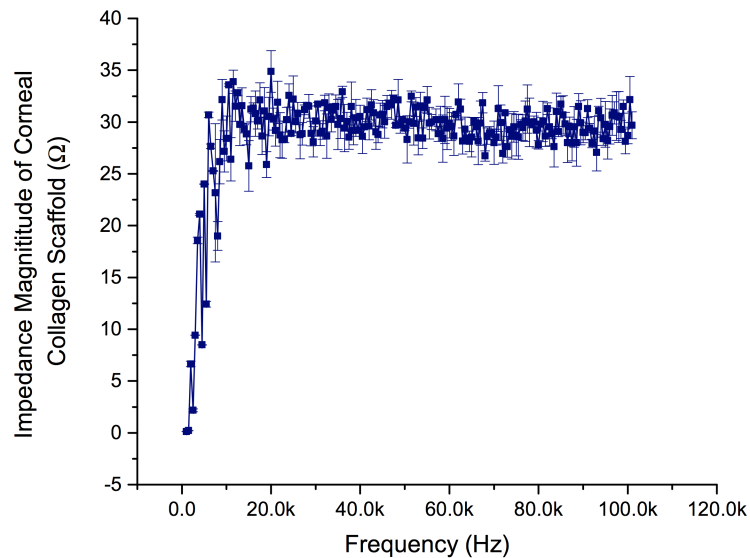


FIGURE 5.19: Measured bioimpedance for Corneal Collagen I Scaffold

450 μm - 500 μm in depth on average and hence a direct contact between scaffold and electrodes would not take place under any circumstances.

5.3.3.4 Feasibility of Using BIS for Cell Density in 3D Environment

The BIS measurement was implemented not only to find its correlation to conventional bioassay, but also to verify its sensitivity in frequency response to cell density in PDMS culture chamber. The BIS measurement took place outside of incubator prior to Alamar-Blue bioassay analysis, as the AlamarBlue bioassay is expected to bring unpredictable offset to BIS measurement. The temperature was kept at 37 degree by employing the ITO-film based heating system. Both 2D and 3D BIS measurement subtract the constant offset provided by scaffold materials. Figure 5.20 a) b) c) d) show the Cole-Cole plot for both A^+ and C^+ cell in 2D and 3D cell culture conditions respectively. The size of the locus for each plot represents the magnitude of bioimpedance of inspected biological sample. It clearly can be seen for 2D and 3D cell cultures, the locus of semi-circle increases with respect to the corresponding increases in cell density. Furthermore, the most sensitive frequency range in response to density change is identified to be within

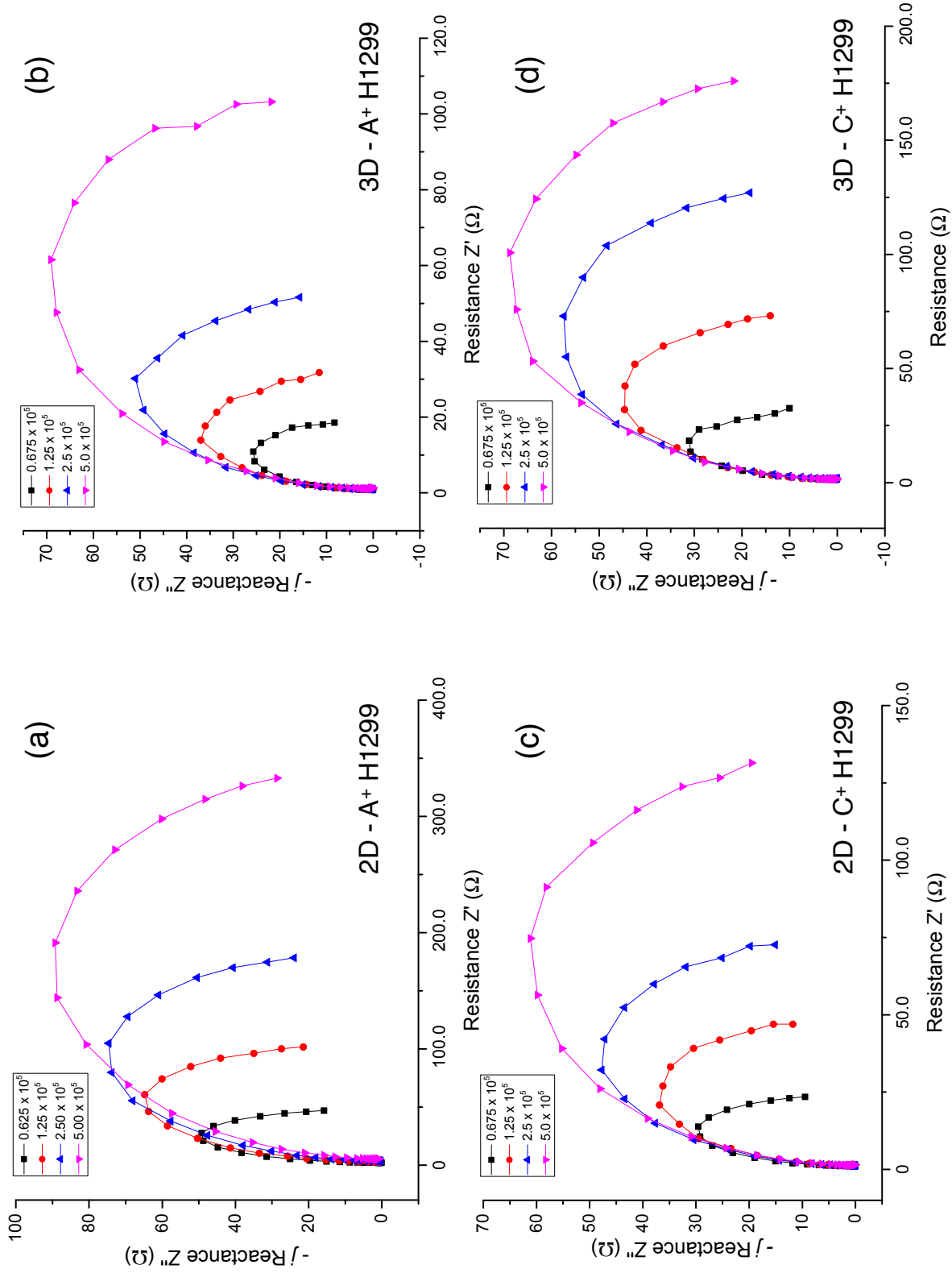


FIGURE 5.20: Cole-Cole Plot for both A^+ and C^+ H1299 Cells in 2D and 3D cell culture environment with Matrigel: (a) A^+ in 2D; (b) A^+ in 3D; (c) C^+ in 2D; (d) C^+ in 3D. Each colour curve represent a bioimpedance frequency response at desired cell density. Black: 0.625×10^5 Red: 1.25×10^5 Navy: 2.5×10^5 and Magenta: 5×10^5 cells/ml

1.5 kHz till 35 kHz as illustrated in the diagrams. Moreover, by comparing the Cole-Cole expression for A^+ in 2D and 3D it is clear that magnitude of bioimpedance in 2D for each density cellular substrate is larger than corresponding impedance of cellular substrate with the same density in 3D environment. In contrast, the magnitude for C^+ bioimpedance does not appear to be significantly different. The 3D cell culture is the

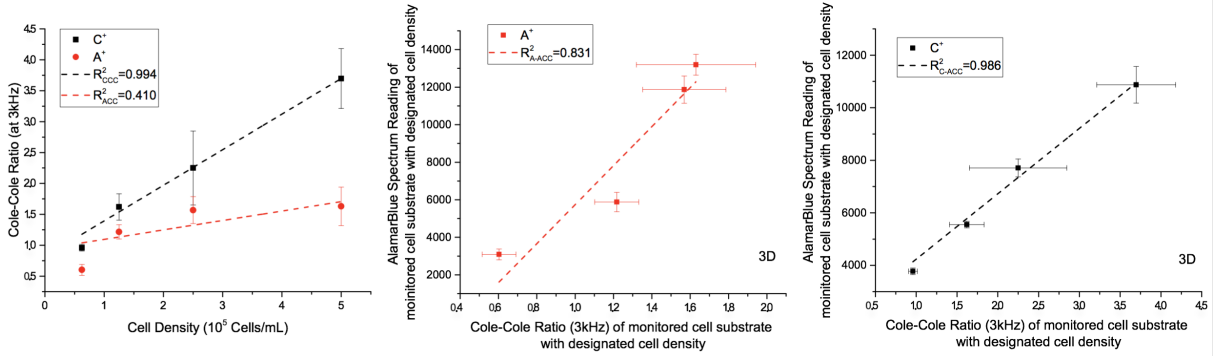


FIGURE 5.21: (a) Cole-Cole Ratio (at 3 kHz) for both C^+ and A^+ H1299 cells with respect to cells' corresponding density in matrigel based 3D cell culture; (b) Linear regression correlation for A^+ between AlamarBlue and Cole-Cole Ratio with different cell seeding density; (c) linear regression correlation for C^+

major interest area for this research as 3D cell culture environment can generally provide more accurate conditions that are similar to the physiological conditions of the human body. Thus the calibration for BIS with cell seeding density is mainly focused on 3D environment. A comparison was made for both A^+ and C^+ Cole-Cole ratio at 3 kHz with respects to their corresponding cell densities in Figure 5.21 (a). The chosen frequency, 3 kHz , was lay in the responsive frequency range, which was illustrated and identified in the Figure 5.20. The Cole-Density plot revealed that C^+ in 3D microenvironment always has a higher cell number compared with A^+ . As it was concluded in Chapter 3, provided that the magnitude of bioimpedance increases, the higher the Cole-Cole ratio at the given frequency, the cell number are said to be increased.

Figure 5.21 (b) and (c) indicate a good linear regression relation between AlamarBlue spectrum reading and Cole–Cole Ratio with designated cell seeding density for both A^+ ($R^2=0.831$) and C^+ ($R^2=0.986$). Thus it is to conclude that the Cole-Cole plot

provides similar information and trends regarding to the metabolic activity of inspected biological sample compared. Though Cole–Cole demonstrated a larger percentage error to the error of AlamarBlue Spectrum analysis, it contains a distinctive advantages over conventional functional assay as it avoids operational interference during culture which could bring contamination to the inspected samples and it does not create an accumulative offset during the repeated inspections.

5.3.4 Feasibility Study of BIS Monitoring for Time-Lapse Based Cell Culture

5.3.4.1 BIS Monitoring in Cell Culture with Optically Different Scaffolds

As it was discussed in Section 5.3.1 (Figure 5.8), the standard deviation for A^+ cell culture with lower ranged haze parameter on 9th day and middle ranged haze parameter on 7th day are crucially large thus only general tendency can be considered in the discussion under such circumstance. Those large standard deviation errors could be introduced by the estimation methodology, which is dependent on the selected image counting; alternatively, it could also be affected by the change of collagen structure in culture medium in the long run. Thus a quantitative method, named Bio-impedance Spectroscopy, to monitor and identify the cellular substrate culturing status is required and it is expected eliminate or at least reduce the operational human error to a controllable level.

Due to the structure of Matrigels can only be sustainable within 144 hours hence it was not fit for time-lapse cell culture purposes and type I Collagen scaffold was chosen in experiments under this section. The fabrication and validation of the system has already been discussed in chapter 3 and obtained data for cell culture with engineered scaffold(9.8 haze value), via proposed 4-Electrodes data acquisition system, is shown in Table 5.1.

Cultured Days	Total Cell Number (x10 ⁵)	Bioimpedance at 3KHz (Ω)	Cole-Cole Ratio at 3kHz
1	0.84	78.96	0.99
3	2.16	156.76	1.32
7	8.52	196.03	1.97
9	15.96	488.62	2.91

TABLE 5.1: In-Situ monitored bioimpedance and analysed Cole-Cole Ratio (3 kHz) for A^+ H1299 Cancer cell seeded onto corneal scaffold (with Haze value 9.8) corresponding to cultured day

The total cell number is estimated by taking the average counting on 5 separate images for each data. Both bioimpedance and Cole-Cole ratio calculation followed the similar procedures as described in Section 3.3.7. The 3 kHz frequency was selected based on the large number of pre-examined experiments and it is also within the active range for the difference of reactance to be identified since it is strictly related to the integrity of overall number of proliferated cells. From Table 5.1, it can be concluded that the overall impedance of the cellular substrate at 3 kHz increases as the estimated proliferation total cell number increases. However, it does not increase at the same rate as the total cell number. A better linearity approximation was obtained by Cole-Cole analysis, shown in Figure 5.22.

The linearity R^2 increased from 0.895 to 0.997 by applying Cole-Cole model to the statistical data. The better linearity was obtained as expected and is mainly due to the electrical property of the biological substrate as explained in Section 3.1.4. The proliferated cells possess more culturing space and causes the extracellular substrate to reduce its cross-sectional area, even though the overall distance travelled by an ion is unchanged. Hence the equivalent conductance decreases and consequently overall resistance increases proportionally to the number of newly proliferated cells. The increase in number for newly proliferated cells can also be regarded as adding a combination of both parallel and series connected capacitors in Cole equivalent circuit model. The

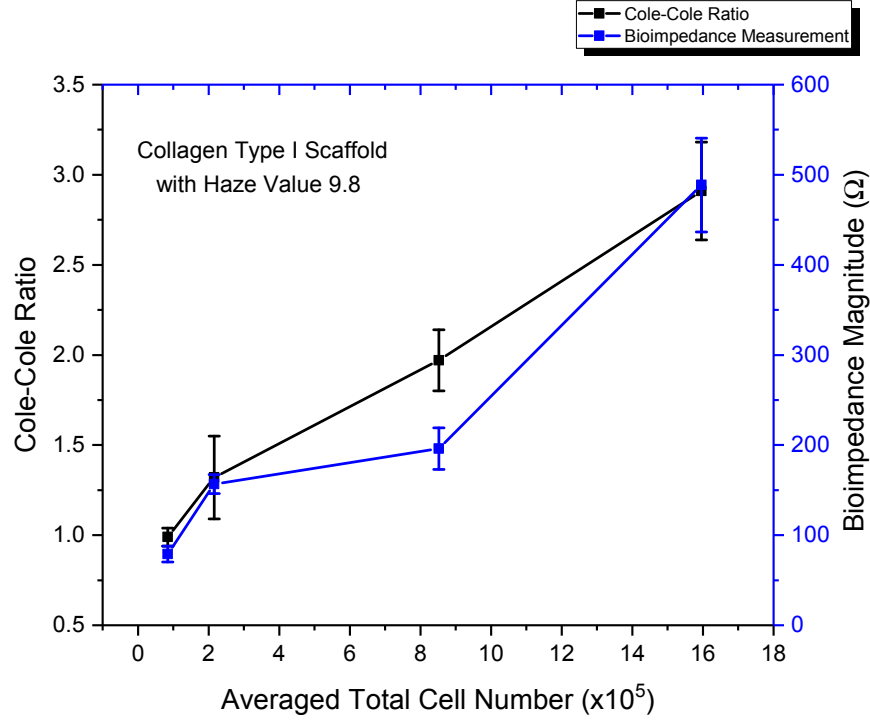


FIGURE 5.22: Cole-Cole Ratio (at 3 kHz) and Bioimpedance magnitude of A^+ H1299 cells with respect to corresponding averaged total cell number over 9 days static 3D cell culture. $R^2_{BIS} = 0.760$; $R^2_{Cole} = 0.993$

equivalent of series capacitance, C_s , is less than any one of the individual capacitors capacitances, whereas equivalent of parallel capacitance, C_p , is the sum of the individual capacitors capacitances and the total capacitance in 3D, C_{total} , is considered as C_s and C_p connected in series. Hence the total capacitance decreases and the electric impedance of total capacitance, known as capacitive reactance of cellular substrate, increases but disproportionately with the number of newly introduced membrane at lower frequency range. The overall impedance represents the root mean square of both reactance and resistance. Thus increasing the overall cell number, through proliferation, will increase both parameters but only one parameter is directly proportional to the total cell number. Thus, the root mean square does not reflect proliferation status accurately. In comparison, the Cole-Cole ratio is a more accurate way to quantitatively identify the cell proliferation status in 3D cell culture with inspected collagen corneal scaffold.

Cole-Cole ratio was determined to be a more accurate analyzing approach for 3D cell

Days	Averaged Total Cell Number ($\times 10^5$)			Cole-Cole Ratio at 3KHz		
	Haze 9 -12	Haze 17-19	Haze 22-26	Haze 9-12	Haze 17-19	Haze 22-26
1	0.84	0.83	0.79	0.99	0.94	1.01
3	2.16	1.89	1.68	1.32	1.20	1.23
7	8.52	3.20	4.04	1.98	1.74	1.54
9	15.96	7.72	6.47	2.91	2.19	1.88

TABLE 5.2: In-Situ monitored bioimpedance and analysed Cole-Cole Ratio (3 kHz) for H1299 A^+ Cancer cell (with density of $1.4 \times 10^5\text{ cell/ml}$) seeded onto corneal scaffold with three optical haze range

culture compared with bioimpedance, and its values versus estimated total cell number for all three ranges corneal scaffold was shown in Figure 5.23 where measured data were summarized in Table 5.2. The image reflects a clear tendency with reduced standard deviation compared to Figure 5.8.

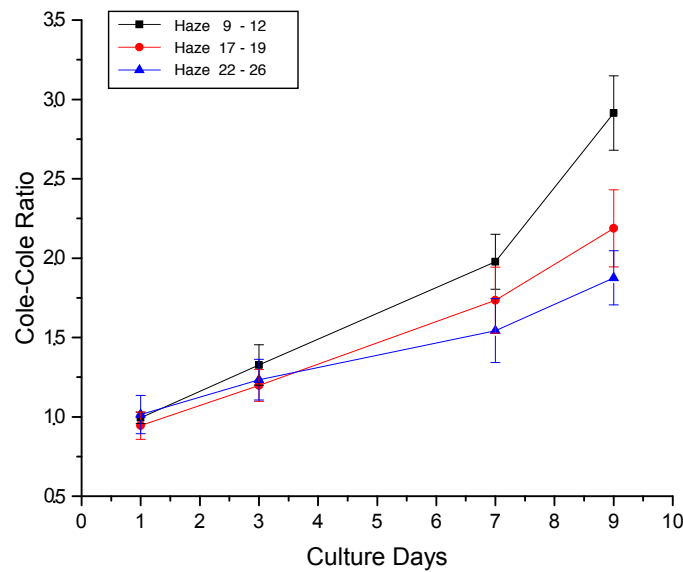


FIGURE 5.23: Cole-Cole Ratio (3 kHz) curve of H1299 A^+ for 3D static cell culture over 9 days where the embedded corneal collagen type I scaffold was pre-inspected and categorized into three different ranges

The analysis was further carried out and the linear approximation between Cole-Cole ratio and total cell number was obtained and displayed in Figure 5.23. It can be clearly stated that as the haze values reduces, an increased linear correlation would appear.

This is because the cellular substrate is more likely to be evenly distributed on the surface of small haze valued corneal scaffold and thus the detected region between referential electrode and measuring electrode, under the previously mentioned circumstances, genuinely reflects the culturing status inside of PDMS microbioreactors compared with situation with higher haze valued corneal scaffold. Moreover, despite the 17 ~ 19 of Haze parameter lies on the boundary of statistically defined QC (quality control) range as stated in Section 4.4, it appears that neither scaffold in 17 ~ 19 haze range nor 22 ~ 26 range facilitated the cell culture with exponential increases in cellular growth. As it is shown in Figure 5.24 (b), the smaller the haze valued scaffold is chosen, the higher Cole-Cole Ratio at a given day, which quantitatively indicates higher the total cell number and metabolic activity, would appeared in the time-lapse cell culture. This suggests that the embedded upper boundary of haze value for quality control purposes should be reduced from the previously stated 21.10 to 16.3 (mean value).

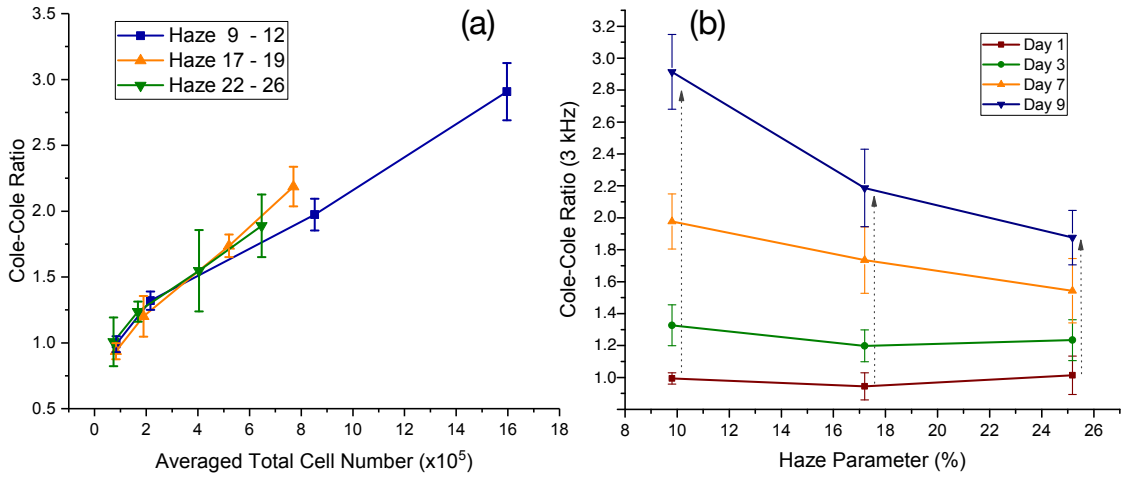


FIGURE 5.24: (a) Linearity approximation between Cole-Cole ratio (3 kHz) and total A^+ cell number; (b) Correlation between Cole-Cole ratio (3 kHz) and optically inspected Haze parameter

The concept of non-destructive inspection was proved by successfully implementing a dozen H1299 cell culture with selected corneal scaffolds into PDMS microbioreactor where the scaffolds were optically categorised into three ranges in haze parameter. The viability of seeded cellular substrate was evaluated both by microscopic observation and

portable on-site bio-impedance spectroscopy device continuously for 9 days. The microscopic observation provided a general tendency that is as the optical haze property increases, the proliferation rate decreases accordingly. In contrast, the bio-impedance and Cole-Cole model provided more accurate information to quantitatively identify the cellular culture status, in terms of total cell number and viability, in PDMS with corresponding haze valued collagen corneal scaffold. The upper boundary of haze value for quality control purposes was re-justified according to the Cole-Cole analysis. The automated ITO heating system was used to provide an homogeneous thermal environment outside of incubator during the spectroscopy measurement.

5.3.4.2 BIS Monitoring of Time-Lapse Based Cell Culture with Different Cells Seeded Onto Selected Scaffolds

The fully inspected Type I Collagen Corneal scaffold were used in this section for 9 days time-lapse cell culture as Matrigel scaffold structure cannot be sustained for more than 6 days in a static cell culture environment. The BIS measurement were taken every two days via proposed tetrapolar data acquisition system where Pt were used for all relevant BIS measurements.

The 9 days time-lapse cell culture was implemented for both C^+ and A^+ H1299 cancer cells with desired cell density of $1.4 \times 10^5 \text{ cell/ml}$ in eight separate samples (e.g. 4 samples for each type of cell). Meanwhile the AlamarBlue bioassay was also operated with same samples ($4 \times C^+$ and $4 \times A^+$) and the assessment was recorded every two days and was carried out in a continuous detection mode. Five microscopic images were recorded on two-day basis for each chamber in order to obtain the averagely counted total cell number.

The microscopic observation revealed the formation of different cell-scaffold interaction between C^+ and A^+ type of cells on 9th culture day which is illustrated in Figure 5.25.

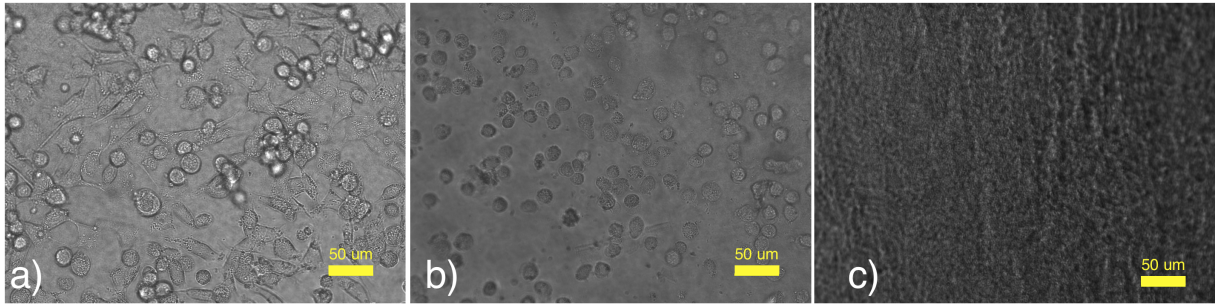


FIGURE 5.25: Microscopic observed cell adhesion on type I collagen scaffold in static cell culture on 9th day a) C^+ ; b) A^+ ; c) Type I collagen scaffold without cell

The C^+ type cancer cell with nature gene expression seems adhere tightly to the underlying type I collagen scaffold. The surface of the C^+ cancer cell seems to come closest to the collagen scaffold and the plasma membrane is specialized at its cytoplasmic face for anchoring stress fibers. In contrast, the A^+ cells revealed a suspension characteristics underlying the same substrate compared with C^+ . They are blebbing (showing membrane bubbles), so are not dying yet but ultimately will if they cannot firmly form focal adhesion bonds with scaffold surface. This is due to genetically modified H1299 cell, by removing RASSF1A gene in the sequence, is expected to be less able to form durable attachments with a surface, since the undergoing micrometer-scale deformation to form an extensive interaction zone allowing molecular contact is unlikely to take place.

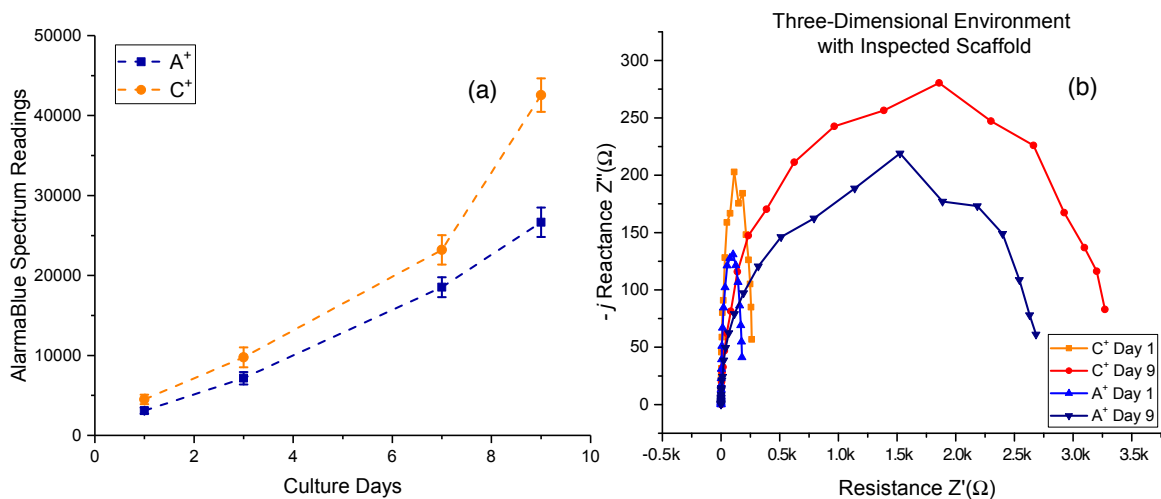


FIGURE 5.26: (a) AlamarBlue Spectrum reading for 3D static cell culture over 9 days; (b) corresponding Cole-Cole plot for C^+ and A^+ cell culture on Day 1 and Day 9

As a result, confluence for C^+ were observed to be higher than A^+ on 9th day, which indicates the higher proliferation rate over time-lapse cell culture. In theory cells use integrin receptors to bind to the RGD groups in the Collagen and Laminin proteins. Because the proteins are clustered, the RGD groups are close together and lots of integrins bind, leading to the formation of a focal adhesion plaque inside the cell. The actin cytoskeleton binds to this and starts a cascade of cell signalling and cell shape change. The lack of communication between extracellular matrix and actin filament protein for A^+ prevented cells from spreading in the first place. Denatured protein on the surface of the vessel or a surface that attracts albumin also slows down spreading. Neutral charge on the surface also affected protein adhesion and reduce the cells ability to spread. Normal surfaces can be treated to a small platform contains a positive or negative charge, and this will allow proteins to electrostatically (non-covalently) bind onto the surface.

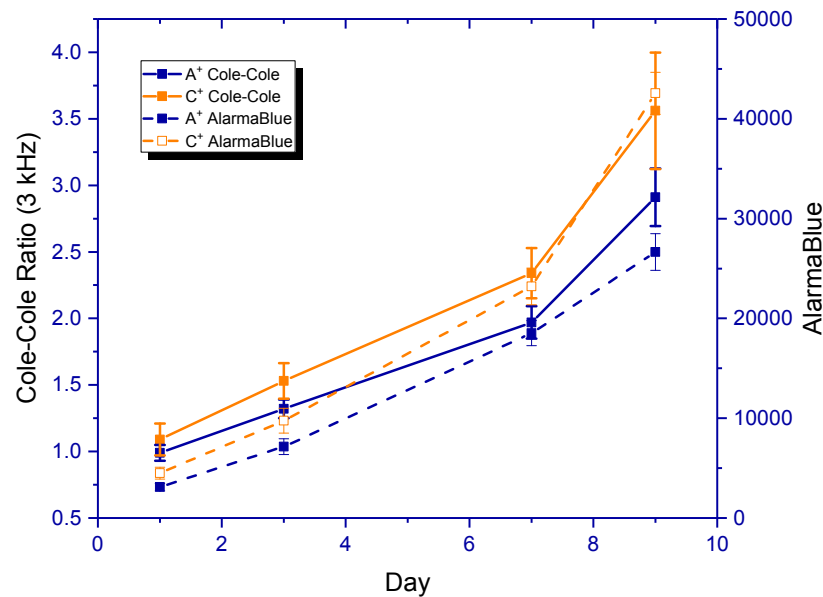


FIGURE 5.27: Comparison of Cole-Cole plot at 3 kHz and AlamarBlue Spectrum readings for time-lapsed 3D cell culture

Although both AlamarBlue assay and Cole-Cole analysis revealed that the C^+ is more proliferated than A^+ in 3D cell culture over 9 days, which is illustrated in Figure 5.26 (a)

and (b) respectively, it does not share the same rate. The constant BIS offset contributed by collagen scaffold and culture medium was subtracted for every single measurement to improve the data's accuracy. There is a clear increase in magnitude of bioimpedance of cultured cells over 9 cultured days. The differences in both BIS and Cole-Cole analysis directly reflects the difference in total cell number; it offers an reliable indication for the growth rate and metabolic activity that is mainly caused by the difference of cell adhesion capability which triggered by genetic modification.

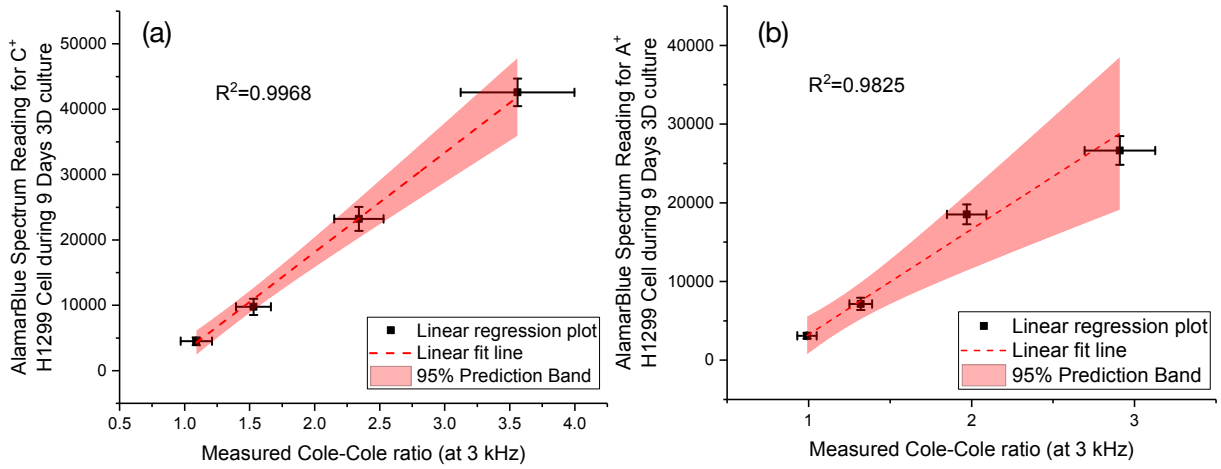


FIGURE 5.28: The normal Quantile-Quantile plot for AlamarBlue Spectrum Reading and measured Cole-Cole value of H1299 cell over 9 days time-lapse static cell culture: (a) C^+ ($R_C^2 = 0.997$); (b) A^+ ($R_A^2 = 0.983$). Prediction Band indicate if the same sampling method was applied to select different sets of Cole-Cole and computed an interval estimate for each Cole-Cole value, we would expect the true population parameter to fall within the interval estimates 95% of the time

Furthermore, the Cole-Cole Ratio Figure 5.27 at 3 kHz is plotted and revealed a similar tendency with respect to the continuously recorded AlamarBlue assay measurement. The further linear regression analysis was carried out and the approximation for C^+ and A^+ was determined to be 0.997 and 0.983 respectively. This proves the concept of using BIS as an alternative methods to monitor a time-lapsed cell culture in 3D culture environment. The confidence band (95%) appeared in Figure 5.28 describes the uncertainty of the obtained data associated within the interval estimate and the overlapped prediction band is to cover prescribed probability of the Cole-Cole value

from future monitoring during cell culture process provided the cell type and scaffold quality remains unchanged. Only small intervals appeared in statistical analysis for both C^+ and A^+ H1299 cell which indicate an excellent repeatability and reliability of Cole–Cole analysis with respect to the well established AlamarBlue Spectrum reading method.

Thus, not only does BIS provide more convenient and non-destructive method for cell viability monitoring and provide quantitative data to indicating the scaffold applicability to a time-lapse cell culture, it can also be used as an alternative tool to monitor the difference of growth the rate that caused by change of cell functions (in this case, it indirectly confirms the the successes of genetic modification for the cancer cell) with excellent sensitivity and reliability.

5.4 Summary

It is hypothesized that BIS will provide a more accurate and non-destructive measurement compared with conventional bioassay method to quantitatively identify the cellular response (includes total cell number, viability and metabolic activity) to its microenvironment in microbioreactor over a time-lapse 3D cell culture.

Firstly to identifying the best selection optical range to facilitate the corneal scaffold's compatibility with cellular substrate, a dozen of cell culture was implemented where scaffolds were selected from three groups with different haze parameter. The cell growth rate were identified to be positive across the all optical ranges which indicate the inspection method for corneal collagen scaffold to be non-destructive. More importantly, statistic indicate growth rate over 9 days of cultured A^+ H1299 cells is highly affected by the haze parameter. Due to the sensitivity of cellular substrate to scaffold optical

property, the new selection bands (0 % - 16.3 %) for haze was therefore established and was lately embedded into control algorithm in automation system.

In order to use BIS technique to monitoring for time-lapsed cell culture with engineered scaffold, the cyclic Voltammetric analysis was initially implemented to ensure no irreversible interface would be permanently created between electrodes and metabolic cellular substrate in 3D environment.

Secondly, to calibrate BIS measurement with respect to seeded cell density for different types of cells in both 2D and 3D, a number of experiments was conducted include live/dead viability assay and AlamarBlue bioassay for metabolic activity monitoring. T-test for AlamarBlue spectrum reading for designated cell density revealed the changes of culture environment from 2D to 3D is significant for A^+ cell ($p < 0.01$) as its metabolic activities reduced by 76% where as C^+ metabolic activities only fluctuated by 4% and is not significantly affected by adapting to different culture environment ($p > 0.01$). By evaluating the BIS offset given by scaffold, the Cole–Cole plot (at 3 kHz) versus cell density offers a similar tendency to the AlamarBlue spectrum reading, but provides better repeatability and reliability as it does not create an accumulative offset during the repeated inspections. Nevertheless we conclude that the system was able to provide statistical data for monitoring cell culture whereas in contrast the live/dead staining methods can only offer a general guidance but lack quantitative determination.

The sensitivity of BIS system combined with feasibility study of 9 days cell culture were conducted with engineered scaffold, where different types of cell includes C^+ (inherits the natural gene) and A^+ (genetically modified by removing RASSF1A in the gene sequence). Both type of cells were seeded onto selected scaffold with most favourable optical property. The metabolic activity of seeded cellular substrate was evaluated by conventional microscopic observations, AlamarBlue spectrum and μ TAS continuously for 9 days. Microscopically observed the genetically modified cell exhibit different cell

adhesion capability and as a result A^+ is less able to form durable attachments in a 3D microenvironment. The measurement from BIS provided a more quantitative data for 3D cell culture compared with conventional methods and thus was proved to be able to monitor the difference in cell culture and the linear regression between AlamarBlue Spectrum Reading and calculated Cole-Cole ratio for C^+ and A^+ in 3D culture were determined to be 0.997 and 0.993 respectively with a narrow interval for both 95% Confidence Band and Predication Band.

Thus, not only does BIS provide more convenient and non-destructive method for cell viability monitoring and provide quantitative data to indicating the scaffold applicability to a 3D cell culture, it can also be used as an alternative tool to monitor the difference of growth the rate that caused by change of cell functions (in this case, it indirectly confirms the the successes of genetic modification for the cancer cell) with excellent sensitivity and reliability.

Chapter 6

Conclusions and Future Work

6.1 Conclusions

The development of microbioreactor platform for cell culturing holds considerable promise in tissue engineering field since the microfluidic device is highly customizable to make its length scales that are well matched to the physical dimension of investigating biological cells. The combination of the complex set of physical, chemical and biological conditions that surround the biological cell, known as the in-vivo cellular microenvironment, are key factors for success of cell culture. The enhancement of physiochemical environmental impact to cell culture and improvement of electrochemical inspections in biomedical practice, together with continuous advances in electronic technology, has encouraged researchers to make the attempts to investigate and develop miniaturized in-situ total analysis system (μTAS) not only provide a more convenient and non-destructive method for cell viability monitoring in comparison to end-point bioassay, but more importantly can also be used as an alternative assay tool, with high precision and sensitivity, to monitor change of biological function during a time-lapsed based 3D cell culture on a environmentally-controlled microbioreactor platform.

The present work thus started with investigation of physiochemical microenvironment control includes thermal distribution and oxygen permeability. An optically transparent ITO-film based heating system with a large heating area was proposed and constructed with the successful attempt to serve as a thermal control system for long term static cell culture outside the conventional incubation area. The improvement of thermal distribution over large area heating element was achieved through characterization and numerical simulations and it was experimentally proved to be compatible with in-situ microscopic observation over the cell culture process through a selective spectrum band. The SH-SY5Y 4 days long-term cell culture showed temperature of culture chamber was controlled within a tolerable error of ± 0.3 °C. The determine growth rate (amount of doubling in one unit of time) over 4 days static cell culture in two thermal controlled environments demonstrated no significant difference, with level of 9.88 ± 1.86 and 11.32 ± 2.00 ($\times 10^{-3}$) h^{-1} for SH-SY5Y cell culture on the ITO-film platform and in a normal incubator respectively. As a whole, within the experimental conditions explored, this evaluation demonstrated that the proliferation rate of the cultured SH-SY5Y on the developed ITO-film based system is consistent with that of in a conventional cell incubator. Results also concluded that an external oxygenate culture medium through microfluidic channels with a precisely controlled laminar flow rate could be helpful to facilitate the cell culture, but is unnecessary since PDMS is a highly gas permeable material.

Based on the limitation of exist functional assay for cell culture, there is high demand to μTAS system that can be fully compatible with microfluidic devices that not only provide a homogeneous physiochemical microenvironment outside of conventional incubator but also be capable to implement a real-time in-situ monitoring, with excellent sensitivity and reliability, for cellular viability, growth rate (total cell number) and metabolic activity in both 2D and 3D cell culture format over a long period of time. This were enabled based on the theory of dielectric property since the electrical properties of a

biological cell were discovered to have close relationships with viability as well as physiological and pathological states.

Hence it was primarily focused on making decision for structure and specification of proposed system-on a chip for bioimpedance spectroscopy (BIS) measurement. The miniaturization of BIS system was achieved by introducing and utilizing the switching matrix array, MT8816EA, and an electric-impedance analyzer, AD5933, to reduce the size, cost and energy consumption of minoring system to facilitate the concept of portability which is considered to be novel.

The initial investigation was carried out for electrode interfacial effect through a number of Cyclic Voltammetry analysis via a three-electrodes monopolar data acquisition system since it was reported to be the major hinder for long term monitoring in biological field. Platinum was experimentally found to be the most reliable material as electrode for long-term static cell culture.

Electronically, it was known to be very challenging for fabricating a portable electronics to provide a reliable and frequency dependent current where its magnitude is bounded within $100\ \mu A$. A feasibility of using Improved Howland Circuit, as part of AFE circuitries, to constantly supply a $93\ \mu A$ frequency dependent current with wide bandpass range was evaluated. The repeatability for frequency response of Improved Howland Circuit was verified with percentage error $\pm 3.7\%$ to acting as a reliable current source. Hence the feasibility of employing a simplified version of EIT to serve as an in-situ bioimpedance monitoring system for long term 2D and 3D cell culture in microbioreactor was assessed. As a result, a portable chip-based real-time in-situ BIS measurement for cell viability analysis was successfully designed and fabricated through integration of electric-impedance analyser chip AD5933, Arduino microprocessor, unique Switching Matrix Array (MT8816EA), and appropriate Analogue Front End circuitry. The fabricated system was attempted to record the bioimpedance change as the viability of

DLD-1 cell were counted, by utilizing hemacytometer, with decreases from 79 % to 65 % in a 24 hours cell culture and a linear correlation between the hemocytometer counted viability ($R^2=0.836$) and its bioimpedance were found with 3 kHz excited current. Further improvements were validated with proposed Cole-Cole formula with an excellent linear regression ($R^2=0.965$) between BIS and viability.

To verify the reliability and sensitivity of realized μ TAS system for 3D cell culture over a period of time, it had been necessary to assessing and controlling the quality of scaffold where the cultured cells were seeded onto. On the other hand the engineered cornea collagen scaffold may be feasible using re-seeding proper human cells onto a decellularized corneal scaffold. So the quality of the corneal scaffold and its interaction to cells can be controlled by assessing the optical property (i.e transparency, haze and total transmittance). Thus an integrated automated collagen corneal scaffold inspection system was proposed with the attempt to serve as a non-invasive and non-destructive high-throughput screening quality control to identify the crucial optical properties for collagen scaffold. A commercially available optical inspection unit, Haze meter, among a few existing inspection technologies was verified to provide an accurate measurement to quantitatively summaries the properties of collagen scaffold over entire inspecting region. With aid from fabricated automation system, the optical inspection successfully eliminated the fluctuated inaccuracy which introduced by human operation errors. As a result, the reliability of system was tested for 200 hours collectively without any major fault. The optical properties include haze and total transmittance were precisely recorded via the integrated automation system and, by utilizing central limit theorem and 3-sigma rule, were statistically analysed and identified the optical boundary conditions to assess the quality of corneal scaffold for potential 3D cell culture. Both Shapiro-Wilk and Anderson-Darling tests revealed an excellent normality of optical properties within selected bands. The high normality with narrow interval for 95% prediction band indicate a high sensitivity and reliability of integrated system for quality control purpose

in a high-throughput screen manner.

To identify the most favourable optical property of scaffold for cell culture in 3D environment, a 9 days time-lapse cell culture with H1299 cancer cell was implemented. The evaluation of determined growth rate illustrated the cultured cells in microenvironment is highly affected by the optical parameter of type I collagen scaffold where its property can be pre-determined non-invasively via optical inspection system in a dried and fully sealed condition. This significant work also reveals that for total transmittance lies between 61.75 % $\sim$ 64.97 % with haze parameter under 16.3 % together would provide the most desired and favourable scaffold in microenvironment for cell culture. Due to the periodic repeating pattern of collagen is very similar to a crystalline structure and the alignment of fibril arrangement, it can be investigated with the most state-of-the-art Multi-Photon Microscope (MPM) followed 3D reconstruction via Imaris software to identifying the material purity and homogeneity inside of collagen material.

With hypothesis that BIS would provide a more accurate and non-destructive measurement compared with conventional bioassay method to quantitatively identify the cellular response (includes total cell number, viability and metabolic activity) to its microenvironment in microbioreactor over a time-lapse cell culture with inspected engineered scaffold. A selected lung cancer cells were partitioned into two forms where C^+ inherits the natural gene and A^+ was genetically modified by removing RASSF1A expression in gene sequence. To calibrate BIS measurement with respect to seeded cell density for different types of cells in both 2D and 3D, a number of experiments was conducted include live/dead viability assay and AlamarBlue bioassay for metabolic activity monitoring. AlamarBlue spectrum reading with designated cell density revealed the changes of culture environment from 2D to 3D causes A^+ cell metabolic activities reduced by 76% where as C^+ metabolic activity is marginally affected by adapting to different culture environment.

It was microscopically observed that genetically modified cells exhibit different cell adhesion capability and hence are less able to form durable attachment with scaffold. In parallel, the miniaturized μ TAS was proved to be capable to detect the metabolic difference for cells with different interaction with scaffold which caused by genetic modification. The linear regression between AlamarBlue Spectrum Reading and calculated Cole-Cole ratio for C^+ and A^+ over 9 days cell culture were determined to be 0.997 and 0.993 respectively with a narrow interval for both 95% Confidence Band and Predication Band and the Cole-Cole formula in BIS measurement demonstrated itself to be a more reliable quantitative analysis method compared with AlamarBlue bioassay results, as it provides better repeatability and does not create an accumulative unpredictable offset during the continuous monitoring.

Thus, not only does the realized μ TAS provide more convenient and non-destructive method for cell viability monitoring, but more importantly can also be used as an alternative tool to monitor the difference of growth the rate and metabolic activity that caused by change of cell functions (in this case, it indirectly confirms the successes of genetic modification for the cancer cell) with excellent sensitivity and reliability in both 2D and 3D cell culture environment over a long period of time.

6.2 Future work

The continuation of the current project needs to be carried out in the following area

Establish a data base for correlation between optical parameter and microstructure of corneal collagen scaffolds. This is due to only 40 samples of decellularized collagen corneal scaffolds being inspected via the newly integrated automation system and needs large number of inspections before the final statistical boundary for quality control can be established. The accurately expressed correlation between inspected haze parameter

via the non-invasive and non-destructive inspection system and proliferation rate in 3D cell culture requires further evaluation in the future.

Validate the new bioimpedance spectroscopy 16-electrodes based EIT system with cellular substrate. Although the on-site BIS measurement system was proved to be able to provide some measure of the cellular activity, the exact nature of the cell-substrate interactions is unclear. Hence an improved system is needed which can monitor specific parameters cellular substrate on a real-time basis. Contemporary system (commercial as well as research) lacks the capability for real-time bioimpedance mapping and parameterization of evolving cell cultures. On the other hand, it is noted that a reduced version of EIT with sixteen electrodes was fabricated and only electronically validated. It is the system designed mainly focused to analyze the cell-cell interaction and electric properties distribution within the microbio reactor culture chamber; nevertheless, the data analysis requires a well-developed cell-mapping and image reconstruction algorithm which is out of the scope in this thesis but it is an emerging field as it is capable for real-time bioimpedance mapping and parameterization of evolving cell cultures includes cell-substrate interactions (e.g. cell-substrate separation) and cell-cell interactions (e.g. tight junctional resistance).

Detailed heat and mass transfer modelling on the ITO film with PDMS microbio reactor:

In chapter two the uniformity of thermal distribution was achieved and evaluated by inducing heat flux and charge carrier pathways on the surface of ITO. The 3D mesh analysis revealed that small heat gradient exists on the surface of conductive ITO-film and hence further heat and mass transfer modelling via Laplace Equations is essential for absolute optimal uniform thermal energy. In addition to the heat distribution, diffusion rate evaluation of gas permeability with PDMS microbio reactor also needs to be addressed and assessed.

In the long term, further investigation is required for following research topics

Corneal tissue engineering: Repopulate human cells or stem cells onto the optimal scaffold to form cornea implant is one of the key for success in corneal tissue engineering and the selection of cells, the integrating of cells and scaffold is also critically needed. The developed technology can therefore be useful for applications in regenerative medicine and clinical field.

Utilization of BIS with stem cell technologies: Bioimpedance spectroscopy as an assay method can potentially be used to detecting the impedance difference to differently genetic expressed cancer cell as the change of cellular proliferation rate is directly triggered by gene modification. However the capability of proposed BIS-based μTAS to directly detect the gene modifications and linking the BIS measurement with RASSF1A gene expression remains unclear and requires further investigation. Furthermore, the BIS method as an assay to monitoring and indicating stem cell differentiation on a non-destructive and real-time basis would be largely benefit for stem cell research.

Appendix A

The Structural Similarity Index

Structural Similarity Index (SSIM) is used for measuring the similarity between two images where an initial uncompressed or distortion-free image as reference[Wang 2004]. it was designed to improve on traditional methods such as peak signal-to-noise ratio (PSNR) which have proven to be inconsistent with human visual perception. The overall index was calculated between two windows x and y of common size $N \times N$ is:

$$SSIM(x, y) = \frac{(2\mu_x\mu_y + C_1)(2\sigma_{xy} + C_2)}{(\mu_x^2 + \mu_y^2 + C_1)(\sigma_x^2 + \sigma_y^2 + C_2)} \quad (A.1)$$

The $\mu_x, \mu_y, \sigma_x, \sigma_y$, and σ_{xy} are the local means, standard deviations, and cross-covariance for images x, y ; $C_1 = (k_1L)^2$ and $C_2 = (k_2L)^2$ are two variables to stabilize the division with week denominator; L is the dynamic range of the pixel-values; k_1 and k_2 were set to 0.01 and 0.03 by default. The formula is based on three comparison measurements between the samples of x and y : luminance (l), contrast (c) and structure (s). The individual comparison functions are:

$$l(x, y) = \frac{2\mu_x\mu_y + C_1}{\mu_x^2 + \mu_y^2 + C_1}; c(x, y) = \frac{2\sigma_x\sigma_y + C_2}{\sigma_x^2 + \sigma_y^2 + C_2}; s(x, y) = \frac{\sigma_{xy} + C_3}{\sigma_x\sigma_y + C_3} \quad (A.2)$$

C_3 was set to be $C_2/2$ by default. The resultant SSIM index is a decimal value between -1 and 1, and value 1 is only reachable in the case of two identical sets of data.

Appendix B

The Charging Current in Electrochemical Measurement

The conditions for measuring the potential difference between a working and a reference electrode can be approximated by an electrical circuit with a resistor, R_s , representing the solution resistance and a capacitor, C_d , representing the double layer at the electrode/solution interface. Such a resistor-capacitor (RC) circuit is shown in Figure B.1. The charging current can be measured by using a voltage step or voltage ramp method.

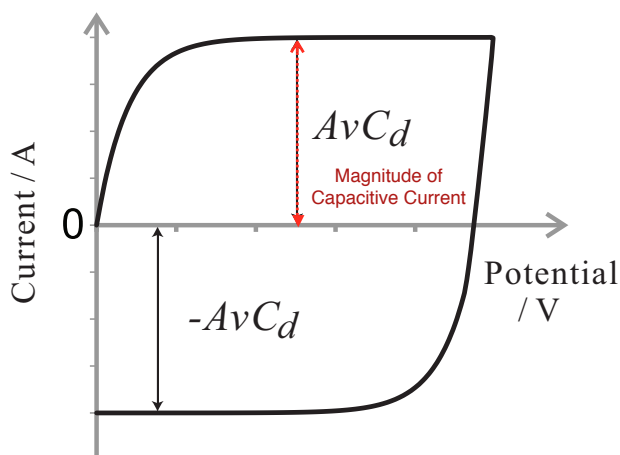


FIGURE B.1: Current-potential plot resulting from a cyclic voltammetry analysis to an RC circuit.

For a general capacitor, the charge, q , is as a function of the voltage across it, E , and is linearly proportional to the electrode area, A :

$$q = AC_d E \quad (\text{B.1})$$

At any time the sum of the voltages, E_R and E_C , across the resistor and the capacitor, respectively, must equal the applied potential:

$$E = E_R + E_C = IR_s + \frac{q}{AC_d} \quad (\text{B.2})$$

Since $I = dq/dt$, so after rearranging Eqn. B.2, it yields

$$\frac{dq}{dt} = \frac{-q}{AR_s C_d} + \frac{E}{R_s} \quad (\text{B.3})$$

In the initial conditions of $q = 0$ at $t = 0$, the solution of Eqn. B.3 is:

$$q = EAC_d[1 - e^{-t/AR_s C_d}] \quad (\text{B.4})$$

By differentiating Eqn. B.4 via dq/dt , the charging current, I , can be obtained as:

$$I = \frac{\Delta E}{R_s} e^{-t/AR_s C_{DL}} \quad (\text{B.5})$$

This equation can be used to determine the solution resistance by using chronoamperometry.

During the linear potential sweep or cyclic voltammetry experiment, the applied potential increases linearly with time at a scan rate ν (V s^{-1}):

$$E = \nu t \quad (\text{B.6})$$

If such a ramp is applied to the RC circuit, Eqn. B.2 yields:

$$\nu t = R_s(dq/dt) + q/AC_d \quad (\text{B.7})$$

If $q = 0$ at $t = 0$, then

$$I = \nu AC_d[1 - e^{-t/AR_sC_d}] \quad (\text{B.8})$$

As the $t \geq AR_sC_d$, the charging current becomes a constant size of νAC_d . Therefore, with known values of scan rates and an electrode surface area, it is able to relate the charging current of an unknown electrode to estimate its electrode surface area, provided the capacitances of these two are approximately the same.

Appendix C

Equivalent Circuit Analysis for Monopolar Data Acquisition System

An equivalent circuit model for measured substrate in Cyclic Voltammetry, under the three electrode data acquisition system, is described in Figure C.1. Based on the design

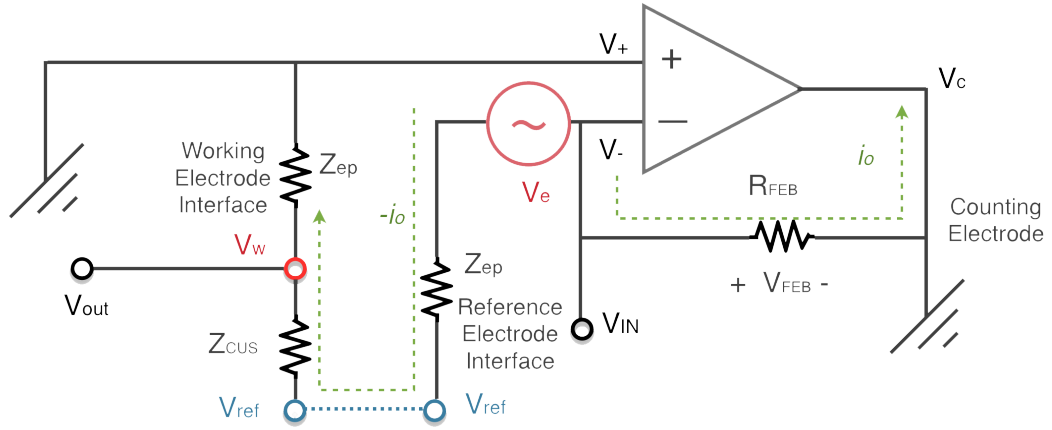


FIGURE C.1: Equivalent Circuit for Monopolar Data Acquisition System.

principle of op-amp and Kirchhoffs current law, it can be clearly stated that

$$V_+ = V_- = V_e = V_{IN}; \quad V_c = 0; \quad V_w = V_{out} \quad (C.1)$$

where the V_c represent potential level on the counter electrode surface and the the output current, i_o , can be expressed as

$$-i_o = \frac{V_{ref} - V_+}{Z_{CUS} + Z_{ep}} \quad (C.2)$$

the Z_{FB} is a feedback resistor embedded into system with determined value and the previously mentioned output current also represent the current pass through the biological inspection sample which can be expressed as

$$i_o = \frac{V_- - V_c}{Z_{FB}} \quad (C.3)$$

By combing Eqn. (C.2) and (C.3), the Eqn. (C.4) can be obtained and rearranged to Eqn. (C.5)

$$-\frac{V_{ref} - V_+}{Z_{CUS} + Z_{ep}} = \frac{V_- - V_c}{Z_{FB}} \quad (C.4)$$

$$V_{ref} = \left(1 - \frac{Z_{CUS} + Z_{ep}}{Z_{FB}}\right) \cdot V_e \quad (C.5)$$

The measured voltage, which defined as V_{out} , is the potential level on working electrode surface and its formula can be derived by Ohms Law

$$V_{ref} - Z_{CUS} \cdot (-i_o) = V_w = V_{out} \quad (C.6)$$

By substituting the Eqn. (C.6) into Eqn. (C.5) and replace V_{ref} , the new Eqn. (C.7) and (C.8) can be obtained

$$\left(1 - \frac{Z_{ep}}{Z_{FB}}\right) = \frac{V_{out}}{V_e} = \frac{V_{out}}{V_{In}} \quad (C.7)$$

$$\left(1 - \frac{Z_{ep}}{Z_{FB}}\right) \cdot V_e = V_{out} \quad (C.8)$$

where Eqn (C.7) represent the transfer function of the measured system. The bio-impedance of the cellular substrate, Z_{CUS} , has been mathematically eliminated in the transfer equation and its magnitude is piratically negligible in comparison to the impedance

of electrode interface based on the aforementioned Stern theory. The dc current is monitored at each dc voltage level and this methodology has been broadly used in electrochemistry field and known as Cyclic Voltammetry analysis.

Appendix D

Equivalent Circuit Analysis for Tetrapolar Data Acquisition System

An equivalent circuit model for measured biological substrate in Bioimpedance, under the four electrode data acquisition system, is described in Figure D.1.

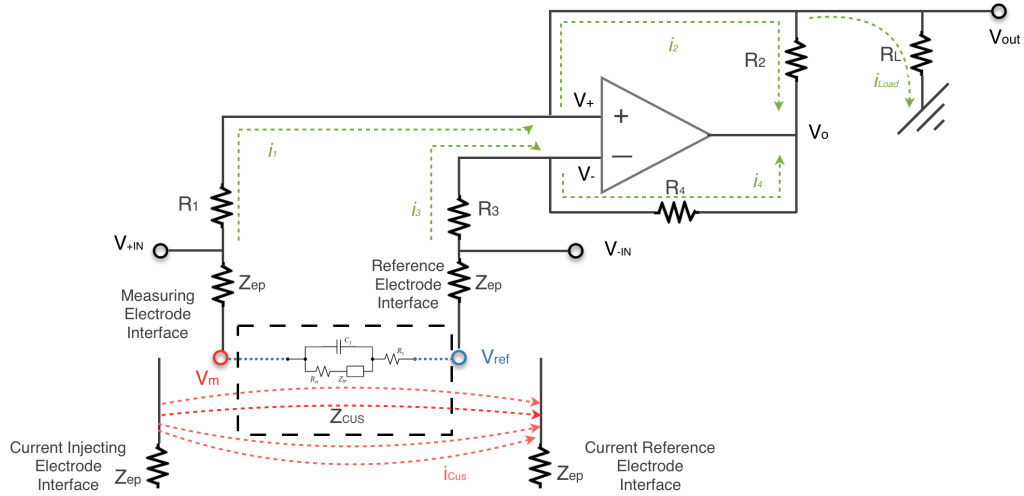


FIGURE D.1: Equivalent Circuit for Tetrapolar Data Acquisition System

Due to the nature of Op-Amp, the i_3 is set to be equal to i_4

$$A = V_+ - V_o = V_- - V_o; \quad i_3 = i_4; \quad (D.1)$$

By considering Kirchoff Current Law at V_o , it therefore obtained equation

$$i_1 = I_2 + i_{load} \quad (D.2)$$

$$\frac{V_{+IN} - V_+}{R_1} = \frac{V_+ - V_o}{R_2} + i_{load} \quad (D.3)$$

By substituting Eqn (1) into Eqn (3)

$$\frac{V_{-IN} - V_-}{R_3} = \frac{A}{R_4}; \quad \frac{V_{+IN} - V_+}{R_1} = \frac{A}{R_2} + i_{load} \quad (D.4)$$

Since the ratio between R_2/R_1 and R_4/R_3 are set to be equal and consequensly substitute the Eqn. (D.1) into equation to eliminat the term R_3 and R_4 .

$$\frac{V_{-IN} - V_+}{R_1} = \frac{A}{R_2}; \quad \frac{V_{+IN} - V_+}{R_1} = \frac{A}{R_2} + i_{load} \quad (D.5)$$

Hence the load current is then expressed as

$$I_{load} = \frac{V_{+IN} - V_+}{R_1} - \frac{V_{-IN} - V_-}{R_1} = \frac{(V_{+IN} - V_+) - (V_{-IN} - V_-)}{R_1} \quad (D.6)$$

By considering the magnituge of measured input voltage is relied on the bioimpdeance value of substrate, thus

$$I_{load} = \frac{(V_m - Z_{ep} \times i_{CUS}) - (V_{ref} - Z_{ep} \times i_{CUS})}{R_1} = \frac{V_{measured} - V_{ref}}{R_1} = \frac{Z_{CUS} \times i_{CUS}}{R_1} \quad (D.7)$$

By rearranging the equation, it is finally obtained the functional equation of data acquisition system

$$V_{out} = i_{load} \times R_L = \frac{Z_{CUS} \times i_{CUS}}{R_1} \times R_L \quad (D.8)$$

Therefore, the bioimpdance of moniotored substrate is calculated as

$$Z_{CUS} = \frac{V_{out} \times R_1 \times i_{CUS}}{R_L} \quad (\text{D.9})$$

It is clearly can be seen that the measured bioimpdance, Z_{CUS} is independent of electrodes impedance, Z_{ep} , and hence provide more accurate results compared with bipolar system.

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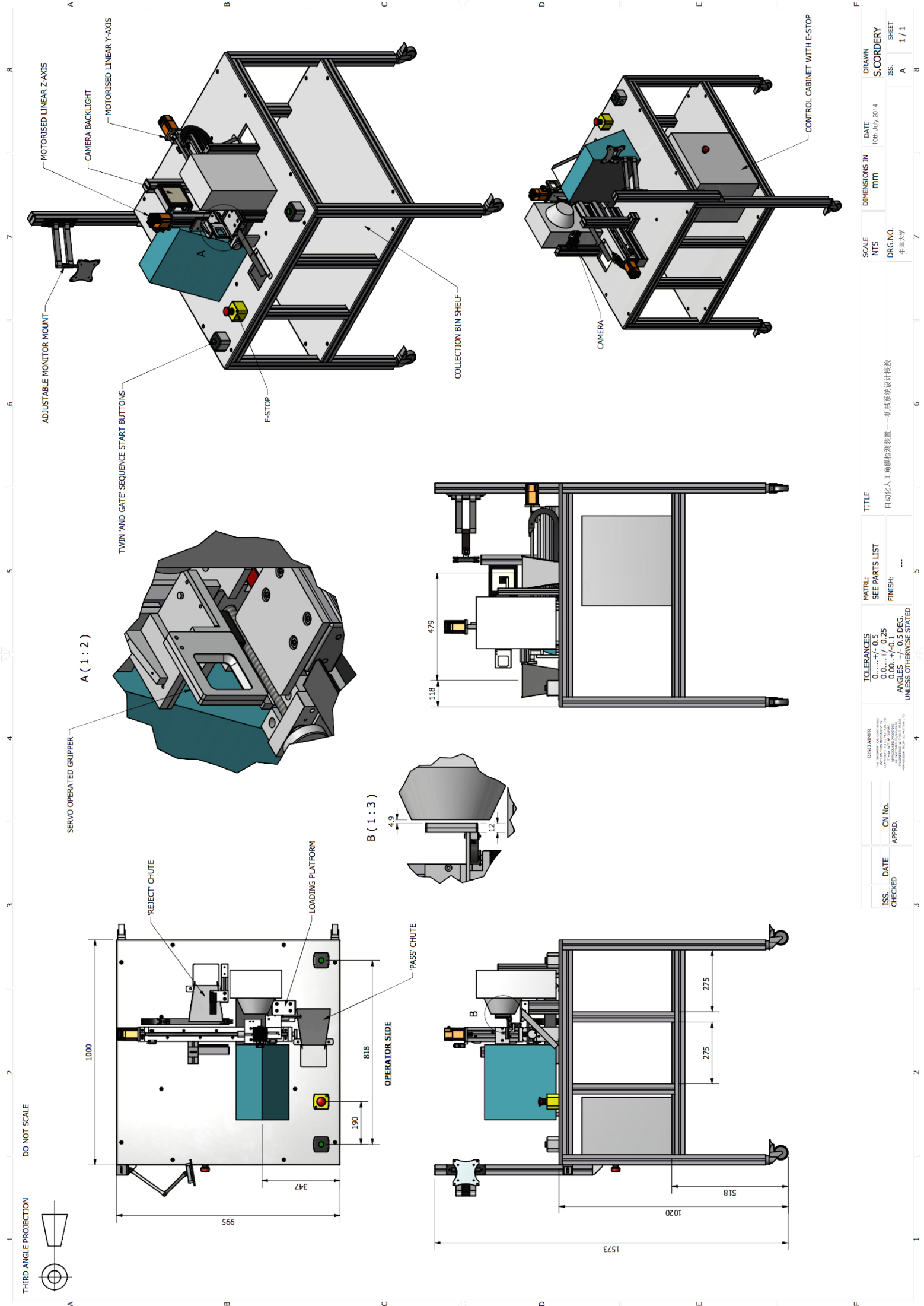
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