MICROFLUIDIC BASED IMPEDANCE FLOW CYTOMETRY-DUAL MICRONEEDLES FOR RED BLOOD CELL DETECTION

MUHAMMAD ASRAF BIN MANSOR

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Universiti Teknologi Malaysia

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DEDICATION

Specially dedicated with love to my wife, Nur Raimi Mohd Abdul Rashid, and our lovely children, Muhammad Aisy Iskandar, Muhammad Kasyfi, and Naira, for their moral, spiritual, and emotional support throughout the journey.

To my parents Mansor Abas & Siti Alfah Mohammed, parent in laws & siblings who shared their words of encouragement to complete this study.

May Allah (swt) shower his blessings upon all of you.

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ABSTRACT

This thesis presents a microfluidic impedance flow cytometry-dual microneedle device for cell detection. Single-cell detection plays a significant role in biomedical diagnostics, such as early cancer cell detection and pathogenic bacteria cells in the blood. The growing need for simple and low-cost microfluidic device fabrication led to the invention of numerous microfluidic-based impedance flow cytometry (IFC) techniques. The current method for impedance flow cytometry technique is limited to an expensive and complex fabrication process of gold microelectrode. Therefore, the IFC-dual microneedle device with a simple design structure and uncomplicated fabrication process for cell detection is presented. The device utilized the two Tungsten microneedles with 25 μm of tip diameter, placed at the half-height of the microchannel as the measurement electrode. The design was characterized and optimized in terms of physical dimension, leakage conditions and sensitivity. The polystyrene (PS) microbeads with three different sizes (5 μm, 7 μm and 10 μm), yeast cells with different concentrations and red blood cells (RBC) were utilized to perform the cell detection of this IFC device. This IFC device was able to detect as low as 1.2×10^4 cfu/mL cells of yeast cells in a solution medium. Moreover, the ratio of the impedance at high frequency vs. low frequency, known as opacity, was used to discriminate between the PS microbeads and RBC. In addition, the proposed device demonstrated that the specific membrane capacitance of an RBC is 9.42 mF/m^{-2}, with the regression coefficients, ρ at 0.9895. Measured results were found to lie in the comparable range with the previous technique (7-14.3 mF/m^2). The presented IFC-dual microneedle device provides an opportunity for simple medical and food safety screening processes in developing countries.

ABSTRAK

Tesis ini membentangkan peranti mikrobendalir sitometri aliran galangan-dwi jarum mikro untuk pengesanan sel. Pengesanan sel tunggal memainkan peranan yang penting dalam diagnostik bioperubatan, seperti pengesanan awal sel kanser dan patogenik bakteria sel dalam darah. Keperluan yang semakin meningkat untuk fabrikasi peranti mikrobendalir yang mudah dan berkos rendah, telah membawa kepada penciptaan pelbagai teknik mikrobendalir berasaskan sitometri aliran galangan (IFC). Kaedah terkini untuk teknik sitometri aliran galangan adalah terhad kerana proses fabrikasi mikroelektrod emas yang mahal dan kompleks. Oleh itu, peranti IFC-dwi jarum mikro dengan struktur reka bentuk yang mudah dan proses fabrikasi yang tidak rumit bagi pengesanan sel dibentangkan. Peranti ini menggunakan dua jarum mikro Tungsten bersamaan 25 μm hujung diameter, diletakkan pada separuh ketinggian saluran mikro sebagai elektrod pengukuran. Reka bentuk ini telah dicirikan dan dioptimumkan dari segi dimensi fizikal, keadaan kebocoran dan kepekaan. Manik mikro polisterina (PS) dengan tiga saiz berbeza (5 μm, 7 μm dan 10 μm), sel yis dengan kepekatan yang berbeza dan sel darah merah (RBC) telah digunakan untuk melakukan pengesanan sel peranti IFC ini. Peranti IFC ini berkebolehan untuk mengesan serendah 1.2×10^4 cfu/mL sel dalam medium larutan sel yis. Selain itu, nisbah galangan pada frekuensi tinggi berbanding frekuensi rendah, yang dikenali sebagai kelegapan, digunakan untuk mendiskriminasi antara manik mikro PS dan RBC. Sebagai tambahan, peranti yang dicadangkan menunjukkan bahawa kapasiti membran spesifik RBC ialah 9.42 mF/m^{-2}, dengan pekali regresi, ρ pada 0.9895. Keputusan yang diukur telah didapati berada dalam julat yang setaraf dengan teknik sebelumnya ini (7-14.3 mF/m^2). Peranti IFC-dwi jarum mikro yang dibentangkan ini memberi peluang untuk proses pemeriksaan perubatan dan keselamatan makanan yang mudah di negara membangun.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

SCA	-	Single Cell Anlysis
GAPDH	-	Glyceraldehyde 3-phosphate dehydrogenase
ESEM	-	Environmental Scanning Electron Microscope
AC	-	Alternating Current
DC	-	Direct Current
RBC	-	Red Blood Cells
PS	-	Polystyrene
PDMS	-	Polydimethylsiloxane
IPA	-	Isopropyl Alcohol
O_2	-	Oxygen
UTM	-	Universiti Teknologi Malaysia
FEM	-	Finite Element Method
3D	-	Three Dimensional
μTAS	-	Micro Total Analysis System
DEP	-	Dielectrophoresis
nQDEP	-	Negative Quadrupolar Dielectrophoresis
IFC	-	Impedance Flow Cytometry
MRI	-	Magnetic Resonance Imaging
HNC	-	Head and Neck Cancer
CAP	-	Cell-Attached Patch
WC	-	Whole-Cell
IOP	-	Inside-Out Patch
ppWC	-	Permeabilized-Patch Whole-Cell -Configuration
EFM	-	Electrostatic Force Microscopy
DNA	-	Deoxyribonucleic Acid
ROT	-	Electrorotation
HeLa	-	Human Cervical Epithelial Carcinoma
DFR	-	Dry Film Photoresist
LOC	-	Lab-on-a-Chip
MEMS	-	Micro-Electro-Mechanical Systems

PCB	-	Printed Circuit Board
EDL	-	Electrical Double Layer
ECM	-	Equivalent Circuit Model
Cr	-	Chromium
UV	-	Ultraviolet
FIB	-	Focused Ion Beam
FIB-SEM	-	Focused Ion Beam - Scanning Electron Microscope
DI	-	Deionized
PBS	-	Phosphate-Buffered Saline
CTC	-	Circulating Tumor Cells

LIST OF SYMBOLS

α	-	Alpha
β	-	Beta
γ	-	Gamma
G	-	Geometric Cell Constant
$\tilde{\varepsilon}$		Complex Permittivity
$\tilde{\varepsilon}_{mix}$	-	Complex Permittivity Of The Mixture Suspending
f_{cm}	-	Clausius-Mossotti Factor
ω	-	Angular Frequency
Φ	-	Volume Fraction
A_e	-	Area of the Electrode
g	-	Gap Between Electrode
$\tilde{\varepsilon}_{mem}$	-	Complex Permittivity of Cell Membrane
$\tilde{\varepsilon}_i$	-	Complex Permittivity of Cytoplasm
R	-	Radius of the Cell
d	-	Thickness of Membrane
C_{med}	-	Medium Capacitor
R_{med}	-	Medium Resistor
R_i	-	Cytoplasm Resistance
C_{smem}	-	Specific Membrane Capacitance
ε_o	-	Vacuum Permittivity
V	-	Voltage
ρ	-	Regression Coefficient
Z	-	Impedance
ΔZ	-	Impedance Change
$\|Z\|high$	-	Impedance Magnitude at High Frequency
$\|Z\|low$	-	Impedance Magnitude at Low Frequency
n	-	Number of Count
C_{dl}	-	Electric Double Layer Capacitance of Electrode
Z_{cell}	-	Impedance Magnitude of Cell

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 Research Background

The cell study has emerged as a distinct new field, as they acknowledged being one of the fundamental building blocks of life. The cell consists of the nucleus and other organelles such as the Golgi complex, endoplasmic reticulum, mitochondria, lysosomes and vacuole enclosed by their own membranes seen in Figure 1.1. Organelles have a wide range of responsibilities for the proper health and functioning of the cell. The cytosol (the intracellular fluid) contains various enzymes, and organic molecules dissolve in water and occupy most of the cytoplasm. The concentrations of charged ions such as sodium (Na+), potassium (K+), calcium (Ca2+), and chloride (Cl) consist in the cytosol cause electrically highly conductive to the cytosol [1]. The cell membrane (also called the plasma membrane) is made up of a phospholipid bilayer that maintains protecting the integrity of the cell structure [1]. The phospholipids contain a hydrophilic polar head (charged phosphates) and two hydrophobic fatty acid (hydrocarbon) chains. It is an excellent electrical insulator and is used to regulate the passive and active transport of materials to and from the cell interior. For that reason, the plasma membranes are most suitable to describe as electrical capacitors due to their insulated property. In addition, the plasma membrane is a very high specific membrane capacitance (~1 μF/cm2 or 0.01 F/m2) due to the thickness of the plasma membrane, which is only (~3.5nm) thick [1].

Moreover, the cells have unique biophysical and biochemical properties to maintain and sense the surrounding physiological environment to fulfill its specific functions [2], [3]. Cellular biophysical properties analysis, such as the electrical, mechanical, optical and thermal characterization of cells, plays critical knowledge to diagnostics, clinical science and the pharmaceutical industry [2]. The identification

of cell types based on the biophysical properties is significant to academic and practical purposes. Most of the biophysical changes of cells show an early sign of disease or abnormal condition to the human body, which make it more reliable to become as potential markers for identifying the cell types such as cancers [4]–[8], bacteria [9]–[11], toxin detection [12] and the status of tissues [13], [14].

Furthermore, the rapid growing technologies (e.g. conventional patch-clamp, dual nanoprobe-ESEM and microfluidics) to investigate the biophysical properties of cells have been invented and developed by researchers in the last decades. The technologies experience the evolution of enhancement every year to meet the requirement and make substantial contributions to the biology and clinical research community [14], [15]. Microfluidic has been widely utilized to provide the optimum benefit to this field because of inadequate and straightforward sample volume preparation, high throughput, and real-time measurement. Hence, the development of microfluidic technologies for single-cell biophysical characterization has been sparked recently.

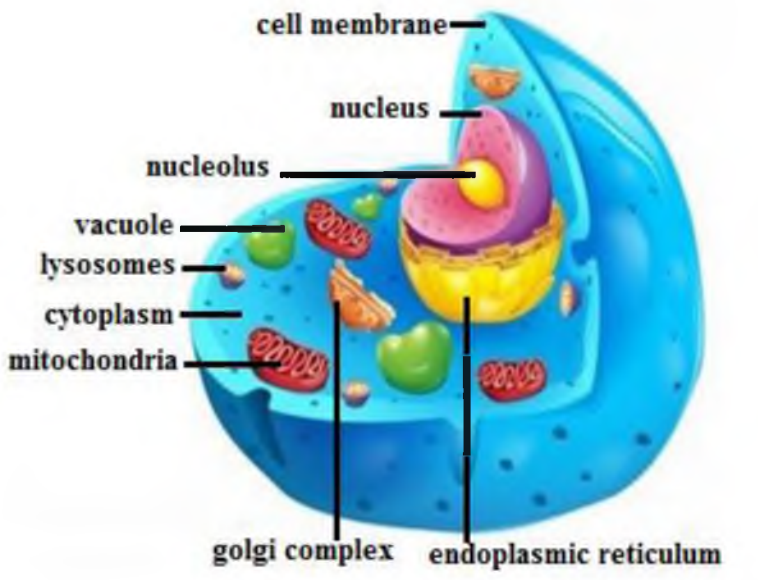

Figure 1.1: The basic structure of the animal cell [16].

1.1.1 Single Cell Analysis

Single-cell analysis (SCA) has become a trend and major topic to engineers and scientists for 20 years to develop the experimental tools and technologies able to carry out the single-cell measurement. In addition, in-depth analysis and more fully described cell differentiation and cancer activities can only be accomplished with single-cell analysis [17]. Several studies reported significant heterogeneity among cells that were previously treated as essentially homogeneous [18]. In conventional methods of cellular analysis, population-based studies have been utilized for cellular processes such as metabolism, motility, cell growth and proliferation. Population methods use averages of cell properties to measure and predict the biophysical and biochemical parameters of the cell. However, this method suffers from inaccuracy measurement and often overlook the essential information available at cell due to the heterogeneity of cell (e.g., specific gene expression level) [19]. One group has described the disconnection between single-cell and average measurements, in which eight individual Jurkat cells treated with siRNA were introduced to the GAPDH gene expression experiment. The results were divided into two distinct groups: partial knockdown (50%) and complete knockdown (0%). While the bulk measurement performed on 50 cells under the same conditions was reduced to 21±4% (n = 4). This value did not represent any result that was measured with single cells.

In this method, the behavior of small populations will be averaged out, and then the behavior of the majority has been used to present a result. For instance, an average of 50% protein expression in a cell population can present either a 100% response in half the cells or a 50% response in all. This can lead incorrectly because the cancer relapse and metastasis may be caused by these small sub-populations in the tumor. For this reason, single-cell studies have been emphasized to provide biologists and scientists to peer into the molecular machinery of individual cells. Single-cell analysis has also been essential to our understanding of some fundamental questions, such as what makes single cells different biophysically, biochemically and functionally.

The accumulation of average and individual cell information from huge populations of cells can offer early detection symptoms of infection or abnormal situations in the human body. For instance, tumor cells discharged into the circulation offer data on tumour growth and therapy efficiency [20]. The presence of irregular red blood cells (RBCs) in the blood can be utilized to detect illnesses such as sickle cell anaemia and polycythemia vera [21]. Single-cell analysis has been a key in the probing of cancer [5], [22], and thus helps doctors to develop a prognosis and design a treatment plan for particular patients.

Technology development in the context of single cells has emerged in the early 1900s through the present micro-and nanotechnology. In the last few decades, rapid development in micro-and nanofabrication technologies has accelerated the potential of sensors to be made in miniature dimensions up to nanoscale, and these developments offer great opportunities for a single cell. Moreover, several miniature sensors have been utilized to characterize the single-cell electrical properties such as impedance, conductivity and others. In this research, the study will focus on the measurement of the electrical properties of a single cell.

1.1.2 Single Cell Electrical Properties Characterization for Cancer Detection

Cancer is abnormal growths of cells caused by genes responsible for regulating cell division is damaged. This may invade tissues and metastasize to the other parts of the body to establish new colonies. The cancer cell can cause significant death of the host if untreated [15]. According to Cancer Research UK, an estimated 8.2 million people died from cancer worldwide in 2012. The most common causes of cancer death are breast, lung, stomach and prostate cancer, which is 50% of all cancers death. The cancer death rate can be reduced by early detection. For example, breast cancer can be detected by using X-ray mammography [23]. However, the capability to detect breast cancer in a thick layer of breast tissue was reduced [24]. Furthermore, ultrasound and magnetic resonance imaging (MRI) also were utilized in breast cancer detection. However, ultrasound has low sensitivity in detecting small and pre-invasive breast cancers from normal tissues due to the

overlapping ultrasonic characteristics of these tissues [25]–[28]. On the other hand, the major problem of MRI is costly and cannot be utilized to diagnose the patient with a pacemaker [29].

In the last few decades, studies on single-cell electrical properties have become a useful method to analyze and characterize the property of single-cell cancer. The cancer cell has a different electrical property (e.g., membrane capacitance and cytoplasm conductivity) than a normal cell. It has become increasingly clear since many researchers have shown their work to differentiate between various cancer (e.g., breast cancer and cervical cancer) cells from the normal cell by utilizing microfluidic technology [5], [30]–[33]. For instance, micro-electrical impedance spectroscopy (μEIS) was utilized to measure the electrical properties of head and neck cancer (HNC) cells [5]. Figure 1.2 shows the phase angle of (HNC) cells at different cancer stages. Based impedance phase value, it can be seen the significant change in electrical properties of cancer cells due to the different cancer cell stages. In addition, this technique may lead to the early cancer detection device. In other words, microfluidic techniques are a handy tool to study the electrical properties of cancer cells for early cancer detection and diagnosis application. Table 1.1 below summarises the previous microfluidic work reported on the cancer cell analysis.

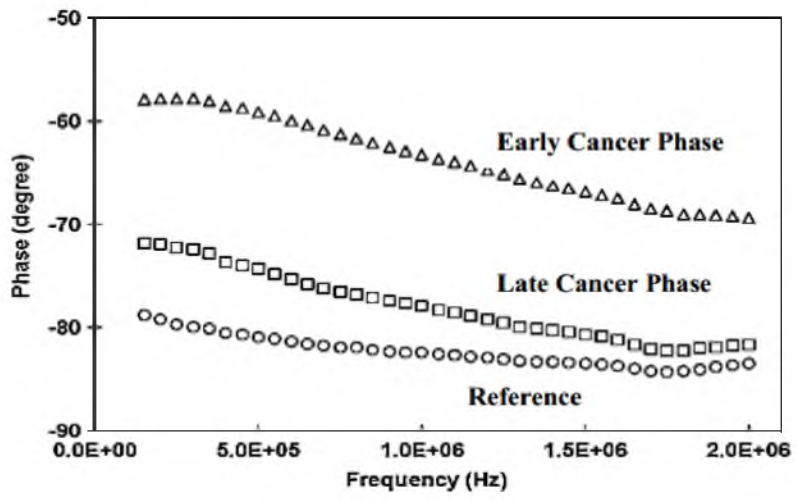

Figure 1.2: Electrical impedance of the different HNC cancer stages [5].

Table 1.1: Summary of research reports on cancer cell analysis by using a microfluidic device.

Cancer Type	Cell line	Summaries
Breast Cancer	MCF-10A, MCF7, MDA-MB-231, MDA-MB-435	Membrane capacitance of the single trapped cell was calculated at 100 kHz to distinguish cancer cell lines from different cancer stages [30]
Cervical Cancer	Hela	The impedance of single-cell trapped by micro pillars was measured at a low-frequency range (1-100 kHz) [34]
Head and Neck Cancer	686LN-M4e, 686LN	Differentiate the poorly metastatic from the highly metastatic cell by observing the phase angle of a single trapped cell [5].
Lung, Breast and Kidney Cancer	H1299, CRL-5803, CCL-185, A549, 95D, 95C, EMT6/AR1.0, 786-O	Specific membrane capacitance and cytoplasm conductivity of single cells aspirated through a constriction channel was characterized [6], [8], [35], [36]
Oral Cancer	OEC-M1	The impedance of cells in the 3D cell culture was measured to monitor the cell proliferation and chemosensitivity [37]

1.2 Problem Background

Single-cell analysis based on electrical properties is important to provide essential data for understanding cellular functions and status. Although highly versatile can be achieved, commercial impedance based flow cytometry are bulky, require complex optics, label and expensive reagents and is highly operator skill-dependent. [38], [39]. The bulky system is hard to introduce as a common device for cell analysis used in the hospital laboratory. More users in clinical research are interested in using a small and portable device.

Microfluidics has emerged as a field that offers vast advantages such as biocompatibility, label-free, low sample volume and simple sample preparation. Microfluidic impedance flow co-plannar electrode, as a well-established technique in single-cell analysis, potentially enabling high-throughput [40]. However, this design has poor sensitivity due to a non-homogenous electrical field across the main channel and requires the complex fabrication process of mircoelectrode. Therefore, the impedance flow parallel electrode was introduced to generate the homogeneous electric field distribution to improve the sensitivity. Current microfluidic based impedance flow cytometry parallel electrode configuration devices experience challenges in terms of fabrication complexity. The Au microelectode fabrication method requires several steps, including standard photolithography[41]. In addition, the standard Au microfabrication fabrication process are expensive (gold electrode) and time consuming, and all the processes are conducted in cleanroom eviroment. Moreover, the two alignment steps are needed for aligning the channel to the electrode pattern and aligning two chips with electrodes together. Precise alignment is needed to make the measurement reproducible [42]. In this view, a simple impedance flow cytometry- dual microneedle device will be designed to achieve the capability to detect and measure the electrical properties of a single cell with a comparable result with previous IFC.

1.3 Research Objective

The objectives of this research are listed as follows:

1. To design and fabricate a simple structure of microfluidic impedance cytometry device for single cell detection.

2. To characterize and optimize the performance of fabricated microfluidic impedance cytometry devices.

3. To test the cell detection functionality of the microfluidic impedance cytometry device using biological and non-biological samples.

1.4 Scopes of Work

The scopes of this research work are as the followings:

1. The commercially available Tungsten needles were utilized as a measuring electrodes due to their high hardness and excellent electrical conductivity.

2. The red blood cell and polystyrene microbeads with a range of diameter between 5-10 μm were used as a target sample for single cell detection. The RBCs were collected from a healthy patients with normal RBC properties.

3. As for single cell detection, the measurement frequency in the range of 100 kHz to 2 MHz was used. Electrode double layer (EDL) effect was negligible in this range.

4. The low flow rate, 6 μl/min was used for single cell detection. The fluid is assumed to be laminar flow fluid.

5. The measuring specific membrane capacitance of red blood cell in the range of 7-14.3 mF/m^2.

1.5 Contribution

In this research, three significant contributions are listed as the followings:

1. A novel integrated dual microneedle-impedance flow cytometry microfluidic for single-cell detection based on impedance measurement has been successfully fabricated. A parallel facing microneedle is integrated with a microfluidic device by utilizing a commercially available Tungsten needle (as a measurement electrode)

2. The dual microneedles are reusable and feasible for electrode gap setting. The variable of electrode gap setting can measure the variety of cell sizes. The function of the device has been tested to measure the presence of a yeast cell concentration, single red blood cell and microbead in suspension.

3. The fabrication cost of this device is reduced compared with the impedance flow cytometry with an embedded electrode. Since the Tungsten microneedle is used as a measuring electrode, the fabrication cost of the *lithography* electrode is eliminated.

1.6 Thesis Outlines

Chapter 2 reviews the existing methods and techniques in the single-cell electrical property analysis from classical platforms to microfluidic platforms. The various microfluidic techniques also have been discussed in this chapter.

In chapter 3, the research methodology that was employed in this study is described in detail. This includes the design and optimization of microfluidic devices and the theory of electrical properties measurements. The chapter also provides the fabrication process of microfluidic microchannel using soft lithography technique and microneedle trim by using Focused Ion Beam (FIB). Lastly, the setup of the experiment is thoroughly explained.

Chapter 4 presents the result of the experiments throughout the research work. The experimental results and analysis are well discussed in this chapter.

Finally, chapter 5 summarizes all the findings of this research work and puts forward recommendations for future works to enhance this microfluidic device in order to perform analysis of a single cell.

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LIST OF PUBLICATIONS

International Journal

No.	Title	Status
1.	Electrical Impedance Spectroscopy for Detection of Cells in Suspensions Using Microfluidic Device with Integrated Microneedles. **Muhammad Asraf Mansor**, Mohd Ridzuan Ahmad, Masaru Takeuchi, Masahiro Nakajima, and Yasuhisa Hasegawa. Applied Sciences. Vol. 7, No.2, Feb 2017.	Published ISI Indexed (IF 2.67) Q2
2.	A Novel Integrated Dual Microneedle-Microfluidic Impedance Flow Cytometry for Cells Detection in Suspensions **Muhammad Asraf Mansor**, Mohd Ridzuan Ahmad, Masaru Takeuchi, Masahiro Nakajima, and Yasuhisa Hasegawa. International Journal of Electrical and Computer Engineering (IJECE). Vol.7, No.3, June 2017.	Published Scopus Indexed
3.	A Simulation Study Of Single Cell Inside An Integrated Dual Nanoneedle-Microfludic System **Muhammad Asraf Mansor** and Mohd Ridzuan. Jurnal Teknologi. Vol.78, No.7, Jan 2016	Published Scopus Indexed
4.	Single Cell Electrical Characterization Techniques. **Muhammad Asraf Mansor** and Mohd Ridzuan. International Journal of Molecular Sciences. Vol.16, No.16, June 2015.	Published ISI Indexed (IF 3.25) Q2

Conference Proceedings

No.	Title	Status
1	The effects of thymus plant extracts on single breast cancer cell morphology in the microfluidic channel. Mohd Ridzuan Ahmad **, Muhammad Asraf Mansor**, Maryam Alsadat Rad, Alan Soo-Beng Khoo, Munirah Ahmad and Marini Marzuki. IEEE EMBS Conference on Biomedical Engineering and Sciences (IECBES 2018), 2019	Published Scopus Indexed
2	A Simulation Study of Cell Separation in Microfluidic Channel Based on Hydrodynamic Principle**.** Mohd Ridzuan Ahmad**, Muhammad Asraf Mansor** and Ida Laila Ahmad. IEEE EMBS Conference on Biomedical Engineering and Sciences (IECBES 2018), 2019	Published Scopus Indexed

Book Chapter

No.	Title	Status
1	Microfluidic Device With Removable Electrodes For Single Cell Electrical Characterization. **Muhammad Asraf Mansor** and Mohd Ridzuan. Handbook Of Single Cell Technology, Chapter 16, Springer Singapore, 2021	Published
2	Microfluidic Device for Single Cell Impedance Characterization. **Muhammad Asraf Mansor** and Mohd Ridzuan. Current and Future Aspects of Nanomedicine. Chapter 6, IntechOpen, 2020.	Published
3	Microfluidic Device With Integrated Microneedles For Detection Of Cells In Suspensions. **Muhammad Asraf Mansor**, Mohd Ridzuan Ahmad, Masaru Takeuchi, Masahiro Nakajima, and Yasuhisa	Published

	Hasegawa. Micro-Nano System Engineering, Vol. 3, Chapter 7, Penerbit UTHM, 2017.	
3	**Muhammad Asraf Mansor** and Mohd Ridzuan. Micro-Nano System Engineering, Vol. 1, Penerbit UTM	Published