Determination of electophysiological properties of Human Leukocyte subpopulations using Lab-on-chip Device

By

Lina Arif Alsharif

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LIST OF SYMBOLS AND ABBREVIATION

AC	Alternating Current
APC	Allophycocyanin
APC/Cy7-	Tandem conjugate
AutoCAD	Drafting and Design computer software used in microelectrode
CD14	Cluster of Differentiation 14
CD16	Cluster of Differentiation 16
CD3	Cluster of Differentiation 3
CD4	Cluster of Differentiation 4
CO2	Carbon dioxide
DEP	Dielectrophoresis
n-DEP	Negative DEP
p-DEP	Positive DEP
24K	24-karat is pure gold used coated microscopic glass slides
3D	Three-dimensional electrode structures
EDTA	Ethylenediaminetetraacetic acid
E-pure	Multistage ion-exchange system)
FACS	Falcon Round-Bottom Tubes. Designed for flow cytometry applications.
FACS	Fluorescence-activated cell sorting
FITC	Fluorescein isothiocyanate
GMCSF	Granulocyte Macrophage Colony Stimulating Factor
GMP	Good manufacturing practices
HIV	Human Immunodeficiency Virus
IL-	Interleukin- family(group of cytokines)

ITO	Indium Tin Oxide
JD	Service providers to many photo-tools
KCl	Potassium chloride
KH2PO4	Potassium dihydrogen orthophosphate
LOC	Lab on chip devices
MACS	Magnetic-Activated Cell Sorting
MATLAB	MATrix LABoratory is a multi-paradigm numerical software
MCSF	Macrophage Colony-Stimulating Factor
Na2HPO4	Di-sodium hydrogen orthophosphate anhydrous
NaCl	Sodium chloride
NK cells	Natural Kller cells
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-Buffered Saline
PerCP	Tandem conjugate
PerCP-Cy5.5-	Tandem conjugate
ROT	Electrorotation technique measure a dielectric properties of cells
RPMI	Roswell Park Memorial Institute (medium used in cell or tissue culture)
T25	Tissue culture treated flasks for cell culture

ABSTRAK

Prinsip dielectrophoresis (DEP) adalah berdasarkan kepada polarisasi dan pergerakan bioparticles dalam medan elektrik gunaan. Perbezaan populasi sel disebabkan oleh daya DEP boleh diplotkan sebagai spektrum DEP. Setiap populasi sel akan mempunyai nilai crossover tertentu yang dipaparkan pada spektrum DEP, menandakan perubahan arah daripada frekuensi-(F-DEP) sebagai kekerapan isyarat arus (AC) meningkat digunakan, sekali gus menjadikan ia alat yang berguna dalam membezakan pelbagai populasi sel. Kajian ini telah dijalankan untuk menentukan sifat-sifat elektrofisiologi sel periferal mononuklear darah (PBMC) dan monosit dengan memberi tumpuan kepada kekerapan crossover menggunakan makmal pada cip (LOC) peranti yang menggunakan microarray dot elektrod. PBMC telah diasingkan dengan teknik centrifugation kepadatan kecerunan menggunakan histopaque ficoll manakala monosit telah dipisahkan dengan kepatuhan plastik. Kemudian, penanda permukaan sel pada PBMC dan monosit dianalisis mengunakan flow sitometri. Sepuluh μl daripada PBMC dan monosit yang dicairkan dalam medium DEP telah ditambah ke dalam spacer peranti LOC. Selepas itu, isyarat arus (AC) dialirkan untuk memerhatikan tingkah laku dinamik PBMC dan monosit sebagai tindak balas kepada daya DEP. Imej-imej ditangkap dan dianalisis menggunakan perisian MATLAB. Imej menunjukkan bahawa monosit memaparkan positive(p-DEP) berbanding PBMC. Di samping itu, bentuk sigmoid di PBMC DEP spektrum konsisten dan crossover berlaku pada 200 kHz. Sementara itu, bagi monosit DEP spektrum, bentuk sigmoid tidak konsisten dan crossover frekuensi dijangka berlaku di antara 100 dan 200 kHz. Kesimpulannya, spektrum DEP kedua-dua PBMC dan monosit adalah berbeza seperti yang dijangkakan kerana perbezaan saiz sel. Perbezaan DEP spektrum boleh digunakan sebagai kriteria dalam pembangunan assay pencirian berasaskan DEP pada masa hadapan.

ABSTRACT

The principle of dielectrophoresis (DEP) is based on the polarization and bioparticles movement in applied electric fields. The differentiation of cell population due to DEP force can be plotted in the DEP spectrum. every cell population have specific passing values that will displayed on the DEP spectra, signified change in the directional of frequency-(FDEP) where the applied alternating current (AC) frequency signal increases, then making it a useful tool in differentiating the various cell populations. The current study was carried out to determining the electrophysiological properties of peripheral blood mononuclear cells (PBMC) and monocytes focusing on crossover frequency using lab on chip (LOC) device which utilise microarray dot electrode. PBMC were isolated by density gradient centrifugation technique using ficoll histopaque whereby monocytes were separated by plastic adherence. Then, PBMC and monocytes were subjected to flow cytometry analysis of cell surface markers. Ten μl of PBMC and monocytes diluted in DEP medium were added into spacer of LOC device. Subsequently, the alternating current (AC) signal was supplied to observe the dynamic behaviours of PBMC and monocytes in response to the DEP force. The images were captured and analysed using MATLAB software. Images showed that monocytes display weaker p-DEP compared to PBMC. In addition, the sigmoid shape of PBMC DEP spectra were consistent and the cross over frequency occur at 200 kHz. Meanwhile for monocyte DEP spectra, the sigmoid shape were not consistent and the cross over frequency expected to occur between 100 and 200 kHz. In conclusion, the DEP spectra of both PBMC and monocytes is different as expected due to the difference in cellular sizes. The different of DEP spectra can be used as a criteria in the development of DEP-based characterizing assay in the future.

CHAPTER ONE

INTRODUCTION

1.1 The immune system: Innate and adaptive immunity

The immune system is defined as the cells and molecules collection that responsible in protection against foreign antigens. The immune systems are composed of innate and adaptive immunity which act as defines system. Innate immunity is front liner in protection against foreign antigens. It is known as a non-specific defines mechanism that encounter antigens immediately or within hours to protect host. The innate immune response unable to recognise the same said pathogen that our body may expose in the future due to lack of memory. The components of innate immune response are epithelial barrier, circulating effector cells, circulating effector protein (Complement and C-reactive protein) and plasma proteins (cytokines). Main cells effecter in innate immunity are neutrophils, mononuclear phagocytes which include monocytes and macrophages, as well as natural killer (NK) cells. Adaptive immunity is antigen specific immune response that have slower temporal dynamic response and the hallmark of adaptive immunity is their ability to memorise that facilitate the host to destroy the antigen effectively when they expose to the similar antigen. The cells of adaptive immunity are T and B cells. Innate and adaptive immune response work in concert, any disruption in either system will expose the host to the foreign pathogen. (Abbas et al., 2007; Murphy et al., 2007; Bonilla et al., 2010; Turvey et al., 2010).

1.2 Peripheral blood mononuclear cells (PBMC)

Consists of deferent blood cells has round nucleus. The component of PBMC are monocytes and the other cell such T cells, B cells, dendritic cells, and natural killer (NK) cells (Koncharevic et al., 2014). The different cell types in PBMC have pivotal roles in the immune system especially in immune mediated diseases (Haudek-Prinz et al., 2012). PBMC have been identifies as surrogate markers of several diseases (Nowak et al., 2010). PBMC can be isolated by technique of the density gradient centrifugation from the whole blood using ficoll histopaque.

1.3 Monocytes

Monocytes derived from bone marrow precursors and reside in blood circulation for a few days before migrate into certain tissue to become various types of macrophages (Van Furth & Cohn, 1968). Monocytes play an important role in tissue repair, homestasis and immune defence system (Ziegler-Heitbrock et al., 2010). Both markers CD14 and CD16 have been used in determination of monocytes subpopulations (Ziegler-Heitbrock et al., 2010). There are three monocytes types according to the following classification; the classical monocytes that with a high expression of the marker CD14 while no expression of the other marker CD16 (CD14++CD16-). Otherwise the non-classical monocytes with high expression of CD16 but low expression of CD14 (CD14+CD16++). Meanwhile, the highly express of CD14 and CD16 low expressed (CD14++CD16+) are in the intermediate monocytes (Ziegler-Heitbrock et al., 2010). Monocytes can be differentiated in vitro under the influence of granulocyte macrophage colony stimulating factor (GMCSF) and IL-4 into dendritic cells (Sallusto and Lanzavecchia, 1994). Also in vitro under the effect of the macrophage colony-stimulating factor (MCSF) that can be converted into macrophages (Martinez et al., 2008).

CHAPTER TWO

LITRERATURE REVIEW

2.1 Cells separation technique

Cell separation is an important technique used in various fields of biomedical and in clinical therapy. The invention of various instruments that capable to sort cells from a heterogeneous starting population into specific populations with high purity facilitates various researches on individual cell types (Tomlinson et al., 2013). This leads to new discoveries in wide array of research areas such as regenerative medicine, HIV pathogenesis and cancer therapy (Guo et al., 2006; Takaishi et al., 2009; Terry et al., 2009). In clinical setting, separation of leukocytes from whole blood by aphaeresis and immunomagnetic separation of haematopoietic stem cells are well known immune therapy method by transferring enriched cell populations in the GMP-grade laboratory into a patient to overcome any immune mediated diseases (To et al., 1997; Handgretinger et al., 1998). Cell separation technique attract many researches interest not only in regenerative medicine fields or limited for tissue engineering interest. Nevertheless in other significant areas such as electrical engineering, materials science also in biochemistry and physics field (Ackerman et al., 2002; Howard et al., 2002; Chan et al., 2008). There are three methods for cell separation are established and available in the market namely adherence, density and antibody binding (Tomlinson et al., 2013). In this section, the outlines of each methodology were described.

2.1.1 Adherence

One of the most simple cell separation technique as after digested or explanted primary tissues the cell can then isolate from that tissues that called cellular adherence . For example, isolation of adherence cells such as monocytes. Following isolation of PBMC using density gradient

centrifugation by ficoll histopaque, the cells were cultured in the T25 flask for two hours in complete RPMI medium. Then, non-adherence cells were discarded to remove any leftover of non-adherence cells. Cells were washed two times with sterile PBS. Following discarding the PBS, the complete RPMI medium was added into adherence cells and stored in 5% CO2 incubator at 370C for certain period of time to let the adherence cells undergo proliferation (Figure 2.1). However, the cells isolated using cellular adherence were low in purity and this technique only applicable if no requirement for isolating certain subpopulations (Tomlinson et al., 2013).

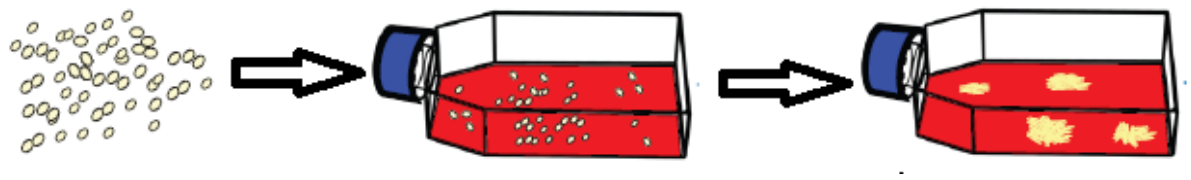

Figure 2.1: Cellular adherence technique. Single cell suspension in complete RPMI medium were added into T25 plastic flask and placed in 5% CO_2 incubator at 37^0C for two hours. Then, non-adherence cells were removed and washed with sterile PBS two times. Following discarding PBS, the complete RPMI medium was added into adherence cells and was incubated for certain period of time to undergo proliferation. Figure and descriptions were adapted from (Tomlinson et al., 2013).

2.1.2 Density

The technique based on centrifugation commonly utilised in the clinical laboratory especially aphaeresis of whole blood for separation PBMC for the leukaemia treatment (Buckner et al., 1969) (Figure 2.2). However, this technique lack of specificity as the densities of each cell

populations is more or less similar which hinder isolation of each individual of cells. These pitfalls can be resolved by centrifugation continuously using variable concentrations of centrifugation medium and different angular velocities (Tomlinson et al., 2013).

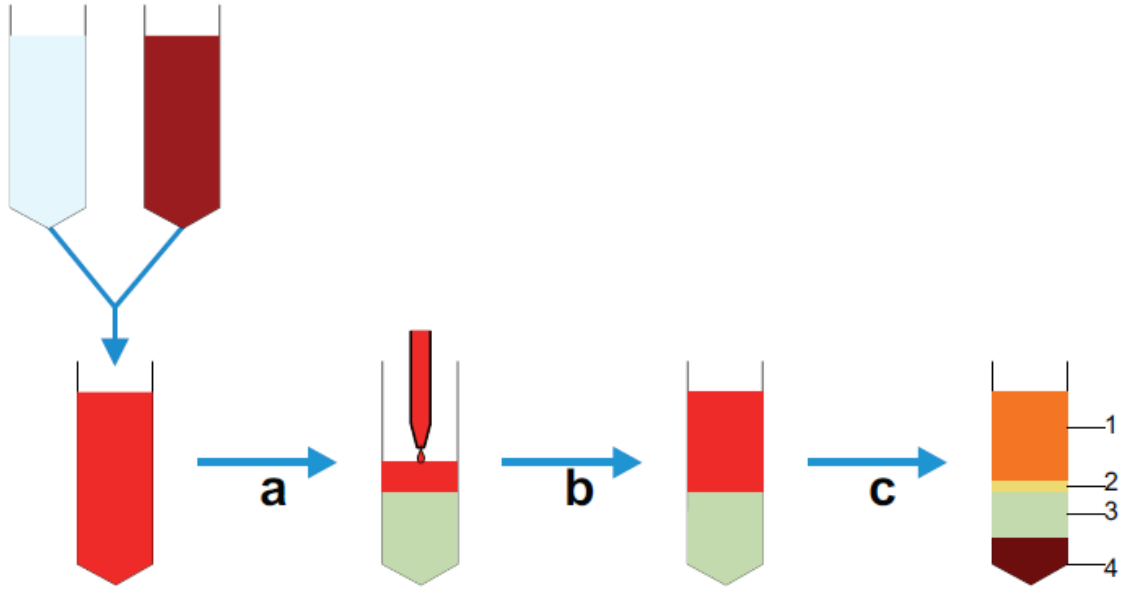

Figure 2.2: PBMC isolation by density gradient centrifugation by ficoll histopaque. (a) Whole blood is diluted with PBS in 1:1 ratio. (b) The diluted blood is then carefully layered on top of ficoll histopaque in 15 ml conical tube. (c) Following centrifugation for 800 g for 30 minutes at 4^0C without brake, four distinct phases is observed; 1- plasma, 2- PBMC, 3- ficoll histopaque and 4- red blood cells. PBMC is carefully aspirated for further downstream applications. Figure and description were adapted from Tomlinson et al., 2013.

2.1.3 Antibody binding

Fluorescence-activated cell sorting (FACS) and magnetic-activated cell sorting (MACS) are two established techniques based on antibody binding methods (Bonner et al., 1972; Rembaum et al., 1982; Miltenyi et al., 1990). FACS and MACS use cell surface antigens against which antibodies are raised to isolate certain type of cells (Tomlinson et al., 2013). Initially, FACS separation require cell suspension to be stained with fluorescence labelled antibodies. The cell suspension is injected into a narrow and rapidly flowing liquid stream by hydrodynamic focusing mechanisms (Tomlinson et al., 2013). The stream consist of the cells separate into individual droplets due to vibration. Before the stream divide into droplets, the flow go through one or more laser beams to ensure the determination of fluorescence pattern of each cell (Tomlinson et al., 2013). The stream is then charged by an electrical charging ring inducing where it convert into droplets based on characteristics of the previous fluorescence analysis (Tomlinson et al., 2013). The charged droplets then fall through an array of electrostatic deflection system that deflect droplets into assigned test tubes or 96 wells plated according to their charge (Figure 2.3). The cells for separation using MACS need to labelled with MACS microbeads and placed in a magnetic field (Miltenyi et al., 1990). The unlabelled cells are go through the MACS column while labelled cells are retained in the MACS column (Miltenyi et al., 1990). Then, MACS column are removed from the magnet, the sterile PBS is added into the MACS column and syringe plunge is used to remove labelled cells (Miltenyi et al., 1990) (Figure 2.4). Both of the FACS and MACS require additional instruments and accessories which increase cost. However, FACS can analysis multiple markers on each individual cell by tagging multiple antibodies with different fluorochromes, whereas MACS technique only able to separate cells based individual markers (Tomlinson et al., 2013). However, the cell sorting process require several hours to be completed while MACS technique can separate cells within one hour (Tomlinson et al, 2013).

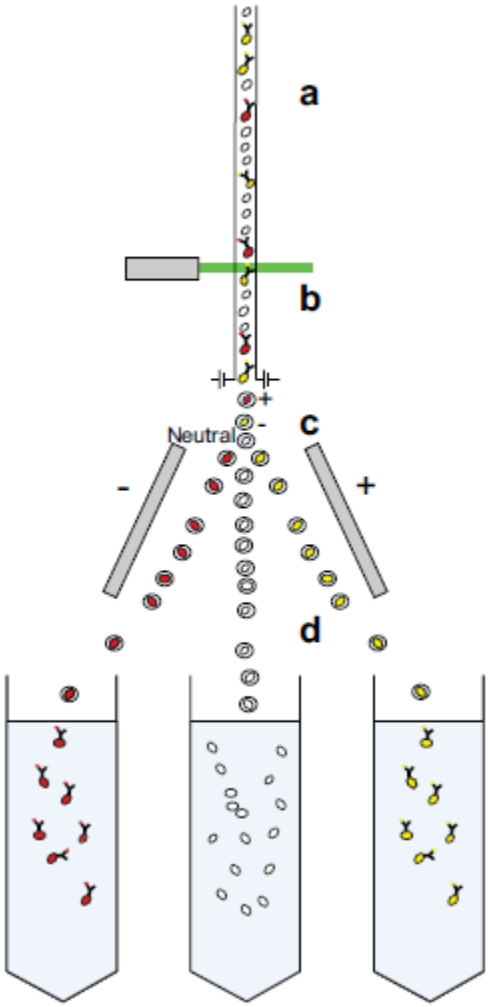

Figure 2.3: FACS separation process. Single cell suspension labelled with fluorescently antibodies are channelled to fluidic system of flow cytometry to enable a continuous stream of individual cells; (b) a light source or laser will detect a flow cells, and the fluorescence intensity of each cell is detected. The cells will be measured either above or below a designated threshold value, and then it is decided whether to collect or not collect each cell. (c) This process is happen when electric charged droplet each cell go through charged deflector plates that sort the cells to the designated collection tubes. Description and figure adapted from Tomlinson et al., 2013.

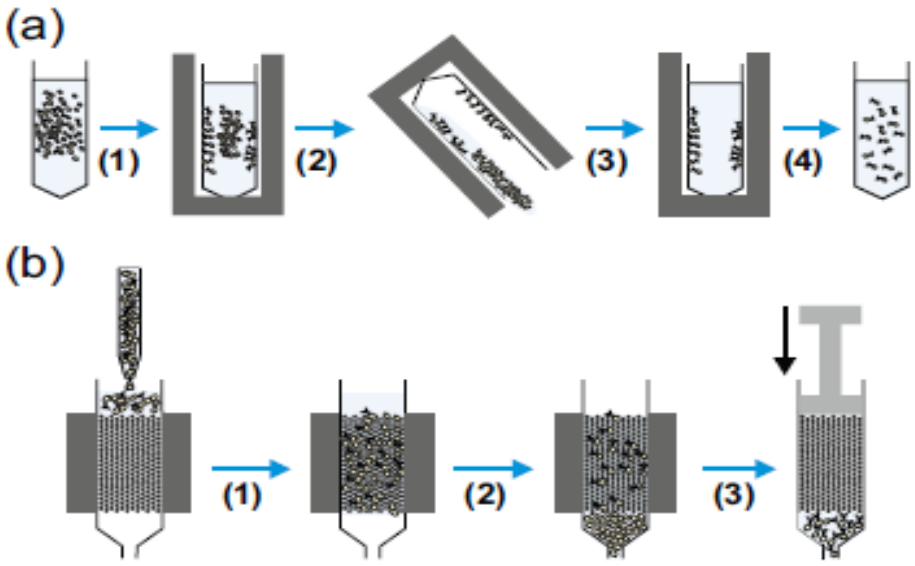

Figure 2.4: MACS separation principle. (a) Tube-based separation is achieved when a magnetically labelled cell suspension held in a conical tube. The tube is then placed in a (1) magnet which attract the magnetically labelled cells to the sides of the tube towards the magnet. The unlabelled cells is discarded by inverting the tube (2) or by aspirating using serological pipette (3), The magnet is removed and the labelled cells were added with medium to obtain a dispersed suspension of labelled target cells (4). (b) Column-based separation is achieved when a magnetically labelled cell suspension is added onto a column held within a magnet, (1) cells then go through the column in which unlabelled cells are eluted while labelled cells are retained in the column. (2) Subsequently, the column is removed from the magnet, and buffer or medium is added onto the column. The magnetically labelled cells suspension is removed from the column by pressing with the plunger (3). Description and figure adapted from Tomlinson et al., 2013.

2.2 Lab on chip (LOC) devices

2.2.1 Dielectrophoresis (DEP)

The interaction between the particle and spatial gradient of the a non-uniform electric field caused movement reaction by a dielectric particle in that electric field. This movement named DEP (Pohl et al., 1971). The polarizability difference between the surrounding medium and the particles itself influence the movement of particles in DEP (Pohl et al., 1978). Positive DEP (p-DEP) response occur when the particles move towards the electrode edge where this region consist of high electric field gradient (Figure 2.5A). When the particles move away of the electrode edge, the response is known as negative DEP (n-DEP) (Morgan et al., 1999) (Figure 2.5B). The particles in DEP respond uniquely to the different frequencies as it also consist of electrical potential (Nurhaslina et al., 2017).

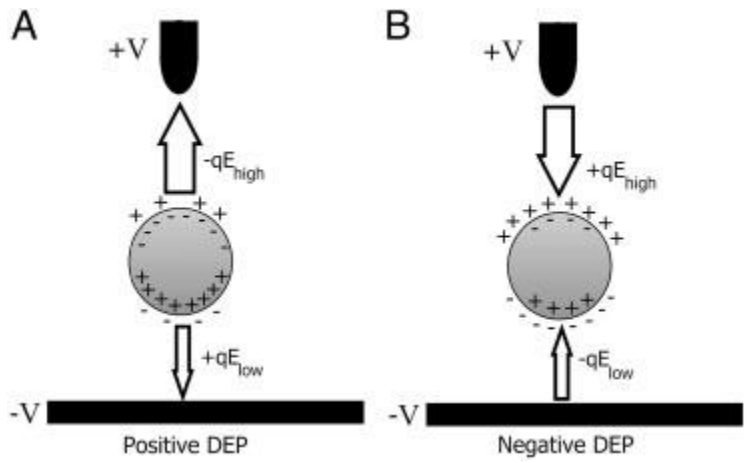

Figure 2.5: DEP force on an induced dipole with the presence of a non-uniform electric field. (A) Positive-DEP; (B) Negative-DEP. (Source: Çetin and Li (2011))

DEP has been widely used in cell lysis research (Young et al., 2012) and to characterize a variety of yeast cells (Patel et al., 2012) and mammalian cells, such as neurons (Jabber et al., 2009), platelets (Piacentini et al., 2011), sperm cells (Rosales-Cruzaley et al., 2013), cancer cells (Chuang et al., 2011), and leukemia cells (Imasato et al. 2010).

DEP effects develop from non-uniform electric field generated by an electrode. During early DEP studies, the electrode structures were fabricated from thin metal wires, needles or plates (Pohl et al., 1977; Jones et al., 1986). However, the advanced microfabrication technologies nowadays helps the fabrication of DEP platform based on microelectrode arrays with the ability to generate strong DEP forces even when small voltages are applied (Hamada et al., 2013; Martinez-Duarte et al., 2013). The well-known structures of electrode are planar and three-dimensional (3D) (Yafouz et al., 2013). In the current study, 4 × 4 microarray dot electrode are used (Figure 2.6) that was fabricated by our team from Universiti Malaya led by Associate Professor Dr Nahrizul Adib Kadri. They fabricated this electrode by using the standard photolithographic processes. Generally, Gold-coated (24K) with microscopic glass slides from Fisher Scientific, Malaysia were cut into two for generating dimensions of 38 × 26 mm2. They used AutoCAD software to design microelectrode geometry whereby the manufacture of the photomask was performed by JD photo-tools (Oldham, Lancashire, UK). The fabrication processes in details have been described by Yafouz et al., 2013. Similar electrode geometry had been used in conducting study by Fatoyinbo, on cell characterisation of homogenous populations. There are several advantages of dot electrode geometry importantly the confined and well-defined region of analysis, effective electric field penetration and an axisymmetrical electric field distribution (Yafouz et al., 2012).

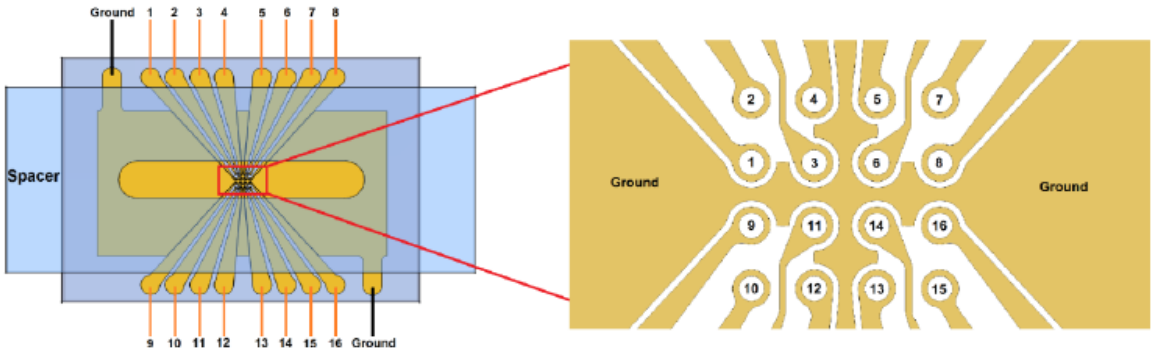

Figure 2.6: 4 × 4 microarray dot electrode. 4 × 4 microarray dot electrode designed by our team from Universiti Malaya in high resolution view. The frequency can be supplied simultaneously to sixteen individual inputs. Figures adapted from Yafouz et al., 2013.

2.2.2 LOC device

Our team from Universiti Malaya have fabricated a novel LOC design as suitable to the microarray dot electrode configuration (Yafouz et al., 2014). The LOC device as shown in Figure 2.7 is a compact of five layers. This microarray electrodes usually use horizontal DEP force which generated by planar electrodes that has parallel arrangement at the same layer (Yafouz et al., 2014). Therefore, the LOC design in the current study placed gold dot microarray electrode on the bottom layer while Indium Tin Oxide (ITO) which acts as the ground electrodes counter electrode at the top, indicates a vertical DEP effect generation by increasing the number of particles experiencing DEP force (Yafouz et al., 2013).

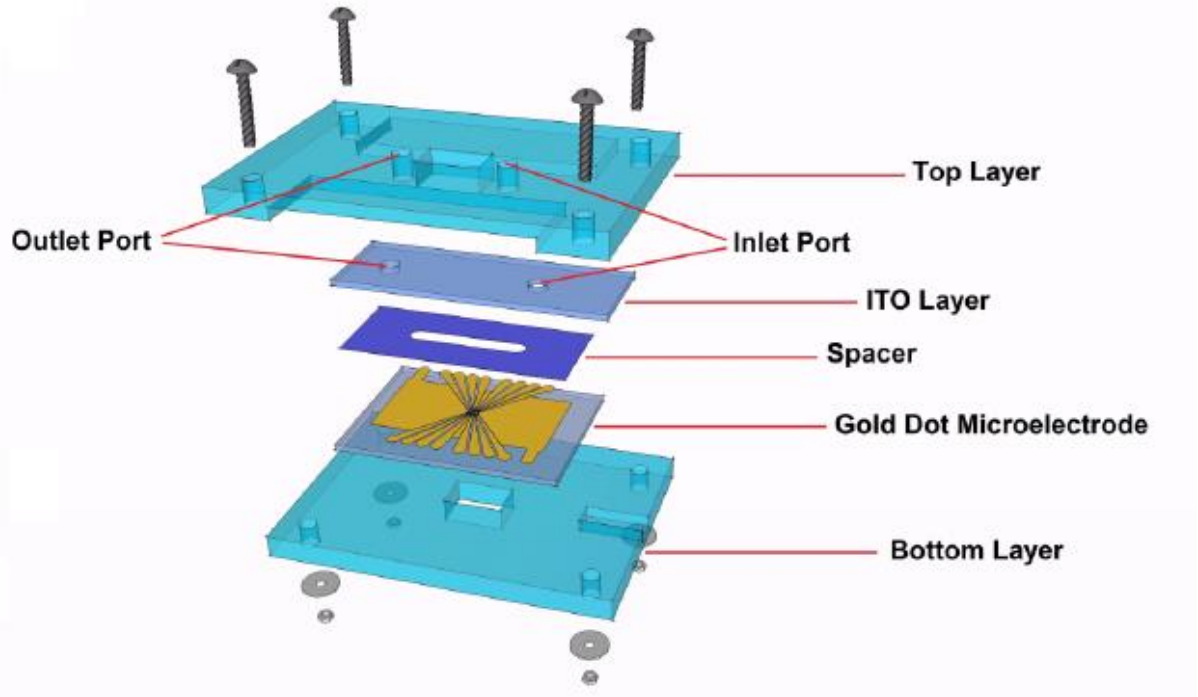

Figure 2.7: The LOC device. Five layers design of LOC device fabricated by our team from Universiti Malaya. Figure adapted from Yafouz et al., 2014.

2.3 Justification and Objectives of the study

2.3.1 Justification of the study

The efficiency of separation and purification technique are very important in the study of leukocyte subpopulation (Yang et al., 1999). Existing cells sorting approaches such as fluorescence activated cell sorting (FACS) (Lambusto et al., 2006), magnetic cells sorting (Kato et al., 1993), and chemically functionally pillar based microchips (Nagrath et al., 2007), have become method of choice for researcher in isolating leukocyte subpopulations. All the mentioned sorting approaches are develop based on known receptors expressed on the surface of the membrane (Salmanzadeh et al., 2013). However, these methods are not effective enough and involve many steps of preparation. In addition, the researcher need to buy expensive equipments such as large magnets, flow cytometers, columns, and centrifuges (Yang et al., 1999). Therefore, by novel approachs, there is a high expectation to develop new sorting devices have identify and selective ability to discriminating cells as compliant to possibility automated and microfluidic their applications (Yang et al., 1999).

DEP influence particle movement following interaction of non-uniform AC electrical fields and field-induced polarisation in particles, has been focused researches nowadays due to great potential for developing the non-invasive separation devices (Huang et al., 1997). A number of studies have been carried out to determine, the human whole blood, dielectric properties (Schwan, 1983), T or B lymphocytes (Bordi et al., 1993; Beving et al., 1994) and erythrocytes (Ballario et al., 1984), all have carried out. And the most recent study on the dielectric characteristics of B- and T cells, monocytes, and granulocytes using electrorotation (ROT) have been performed by Yang et al in 1999.

Our team from Universiti Malaya have fabricated a microarray dot electrode which have several advantages and suit with LOC device. In order to develop LOC device based sorter, it

is very important to initially characterise the differences in the dielectric properties of human leukocyte subpopulations.

2.3.2 General Objective

To determine the electrophysiological properties of Human Leukocyte subpopulations by using lab-on-chip device.

2.3.3 Specific Objectives

1. To isolate and characterise PBMC and monocytes by flow cytometry.
2. To determine the electrophysiological properties of PBMC and monocytes by using LOC device by focusing on crossover frequency.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials and chemicals

3.1.1 Chemical and reagents

All chemicals and reagents used in this study are listed in Table 2.1.

3.1.2 Consumables

All commercial kits and consumables used in this study are listed in Table 2.2.

3.1.3 Enzymes and antibodies

Enzymes and antibodies used in this study are mentioned as list in Table 2.3.

3.1.4 Laboratory apparatus and equipment

The apparatus and equipment used in the laboratory for this study are listed in Table 2.4.

3.1.5 Computer application programmes and software

Table 2.5 lists all the computer application programmes and software used in this study.

Table 3.1 List of general chemicals and reagents

Name	Supplier
di-potassium-EDTA anticoagulant	LP ItalianaSpg., Italy
DEP experimental medium	Sigma-Aldrich Co., USA
Ethanol (absolute)	BDH Chemical, UK
Fetal Bovine Serum	Gibco BRL, UK
Ficoll Histopaque	Sigma-Aldrich Co., USA
Penicillin and Streptomycin	Gibco BRL, UK
Phosphate buffer saline (PBS)	Sigma-Aldrich Co., USA
RPMI 1640	Gibco BRL, UK
Staining buffer	BD Bioscience, USA

Table 3.2 List of consumables

Name	Supplier
Centrifuge tubes (15 ml and 50 ml)	Becton Dickinson, USA
FACS round bottom tubes	Falcon Labware, USA
Syringe 20 ml	Becton Dickinson, Singapore
Tissue culture flask (25 cm^2)	Costar, USA
Top infusion set (23 ¾ G needle)	Meditop Sdn. Bhd., Malaysia

Table 3.3 List of antibodies

Name	Supplier
FITC-conjugated human anti-CD3	BD Biosciences, USA
APC-conjugated human anti-CD4	BD Biosciences, USA
APC-Cy7-conjugated human anti-CD8	BD Biosciences, USA
PerCP-Cy5.5-conjugated human anti-CD14	BD Biosciences, USA
PerCP-conjugated human anti-CD19	BD Biosciences, USA

Table 3.4 List of laboratory apparatus and equipment

Name	Supplier
Axiostar Plus Light Microscopy	Carl Zeiss, USA
Autoflow CO_2 Water-Jacketed Incubator	Nuaire, USA
Centrifuge 5810R	Eppendorf, Germany
E-pure (multistage ion-exchange system)	Barnstead, USA
Esco® Airstream Class II Biohazard Safety Cabinet	Esco Micro Pte. Ltd., Singapore
FACS Canto System	Becton Dickinson, USA
Function generator	Textronic, USA
Leica inverted microscope	WetzlarGmBH, Germany
Memmert incubator	Bachoferlaboratoriumgerate, Germany
Neubauer Improved Hemacytometer	Hirschmann EM Technicolor, Germany
Vortex 2-Genie	Scientific Industries Inc., USA

Table 3.5 List of computer application programmes and software

Name	Supplier
FACS DIVA™	BD Biosciences, USA
Microsoft Office	Microsoft Corp., USA
MATLAB	MathWorks, USA

3.2 Preparation of general solutions and buffers

3.2.1 Phosphate buffer saline (PBS)

To prepare the PBS, 8.0 g sodium chloride (NaCl),1.15 g di-sodium hydrogen orthophosphate anhydrous (Na_2HPO_4) 0.2g potassium chloride (KCl) and 0.2 g potassium dihydrogen orthophosphate (KH_2PO_4), were dissolving in 800 ml deionised water then made up to the 1000 ml final volume with adjusted pH to be 7.4. The buffer had been autoclaved at 121^0C (15 minutes) then stored at 4^0C for 6 months.

3.2.2 Ethanol (70%)

This ethanol solution was prepared by mixed 30 ml of deionised water and 70 ml of absolute ethanol then stored at room temperature.

3.3 Cell culture work

3.3.1 Reagents for cell culture

a) RPMI 1640

One litre of RPMI medium was prepared by dissolving 10.4 g of RPMI 1640 powder and 2.0 g sodium bicarbonate in 800 ml deionised water. The pH was adjusted to 7.2 with 1 M HCl and the final volume was made up to 1 L by adding deionised water. The solution was filter-sterilised with 0.2 μm disposable filter unit and stored at 4^0C until use.

b) Heat-inactivated foetal bovine serum (FBS)

A bottle of 100 ml sterile FBS was placed into a 56^0C water bath for 30 minutes to heat-inactivate the serum. The serum was then aliquoted into sterile 15 ml tubes and stored at -20^0C.

c) Penicillin/ streptomycin stock solution

An antibiotic stock solution containing 10,000 U/ml penicillin and 10 mg/ml streptomycin was prepared by dissolving 0.61 g Penicillin-G and 1.0 g streptomycin in 100 ml PBS. The solution was filter-sterilised and then aliquoted into 15 ml tubes before storing at -20^{0}C.

d) Complete growth medium

A complete growth medium was prepared by adding 10 ml FBS and 1 ml antibiotic (penicillin/streptomycin) stock solution to 89 ml RPMI 1640 medium (10% FBS, 100 U/ml penicillin and 100µg/ml streptomycin). The medium was stored at 4^{0}C.

3.3.2 Culture condition

Cells were seeded in 25 cm^2 cell culture flasks containing complete growth medium and maintained in a humidified incubator at 37^{0}C in an atmosphere of 5% CO_2 and 95% air.

3.4 Peripheral blood collection

40 ml human peripheral blood was obtained from a healthy volunteer by a qualified medical personnel using a 23 G x ¾ needle. The blood was transferred into a 5 ml K_2EDTA tube as soon as possible to avoid coagulation. Blood collection has prior approval from the USM Ethics Committee (USM/JePeM/140384) and collected after informed consent was obtained.

3.4.1 Isolation of peripheral blood mononuclear cells (PBMC)

The uncoagulated blood (40 ml) was diluted 1:1 in PBS and carefully layered over 3 ml ficoll-histopaque in a 15 ml centrifuge tube before being centrifuged at 800 g for 30 minutes at room temperature. After centrifugation, the opaque interface containing mononuclear cells was carefully aspirated using a sterile pasteur pipette and transferred into a clean 15 ml centrifuge tube. The tube was centrifuged at 800 g for 10 minutes at room temperature. Followed by

discard the supernatant then gently mixed and added 2 ml PBS. Following centrifugation at 800 g for 5 minutes and after discarded the supernatant the remain red blood cells were lysed in 5 ml ACK lysing solution at room temperature for 5 minutes. After being centrifugatied at 800 g for 5 minutes then discarded the supernatant the remain cell pellet was resuspended in 1 ml PBS. Cell Concentration of the cell was determined using a haemacytometer. The population of CD3, CD4, CD8, CD14 and CD19 were analysed using flow cytometer.

3.4.2 Isolation of monocyte population by adherence

10-15 x 10^6 PBMC per flask were seeded in T25 cell culture flasks, and left to adhere in a 5% CO^2 incubator for 2 hours in complete RPMI medium. Then, non adherent cells were discarded and washed with 3 ml sterile PBS for two times to completely removed non adherence cells. Subsequently, 2 ml of complete RPMI were added into the flask and were carefully scrapped off the adherence cells by using a cell scraper. The removed adherence cells were transferred into a new 15 ml tube. The flask was then observed under inverted microscope to see any remaining cells. If the cells still available, the complete RPMI medium were added and the scrapping process were repeated again. After the adherence cells were completely removed, the tube was centrifuged at 800 g for 5 minutes at room temperature. Following centrifugation, the supernatant were discarded and the cell pellet was resuspended with 1 ml complete RPMI medium. The population of CD3, CD4, CD8, CD14 and CD19 were analysed using flow cytometer.

3.5 Flow cytometric analyses

This analysis was carried out to ascertain the identity and efficiency of cell separation for PBMC and monocyte population. Each population was subjected to flow cytometric analyses using listed fluorescence-labelled antibody pairs (Table 2.3). 5 µl fluorescence antibodies were used to stain 100 µl of cell suspension. Following 30 minutes incubation in the dark, 2 ml staining buffer was added into each tube and centrifuged at 800 g for 5 minutes. The supernatant was discarded before 300 µl staining buffer was added into each tube. Each preparation was then immediately analysed using the FACS Canto flow cytometry and FACS DIVA^{TM} program.

3.6 DEP theory, DEP device and operation

The DEP theory, DEP device and operation were described by Bashar et al. (2016).

3.7 Experiment set up illustrated in Figure 3.1.

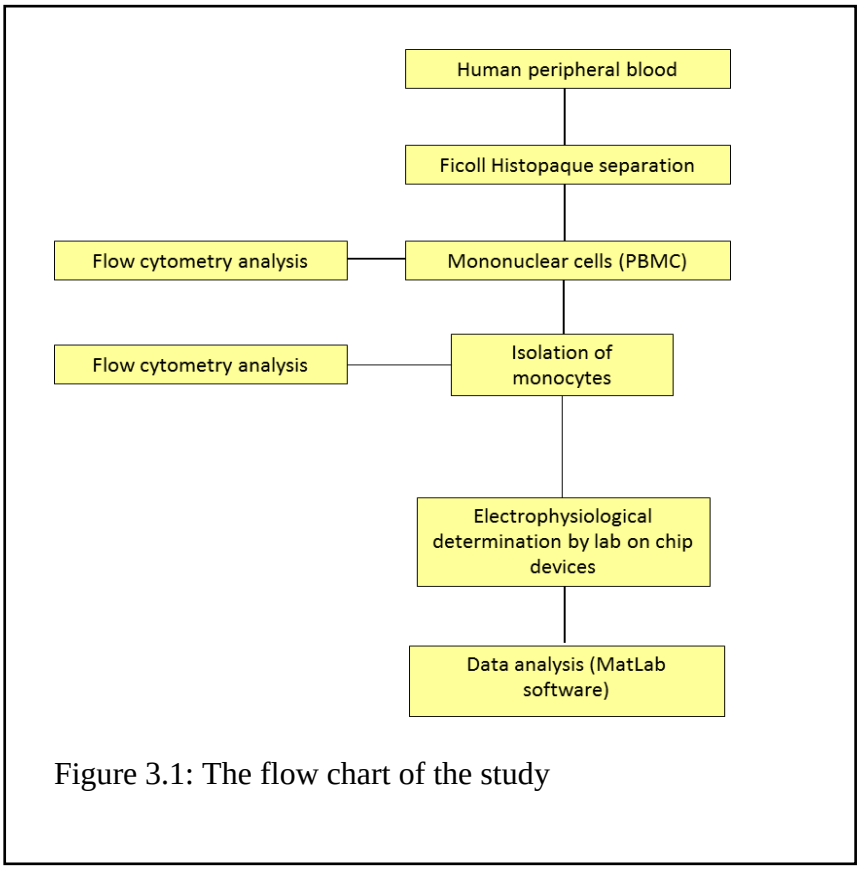

Figure 3.1: The flow chart of the study

3.7.1 Cells preparation

PBMC and monocytes were prepared as described in section 2.4.1 and 2.4.2 respectively. PBMC and monocytes were resuspended in 2 ml of DEP experimental medium that contained 280 mM D-mannitol (Sigma Aldrich, USA). Then, the cells were washed twice with DEP experimental medium by centrifugation at 800 g for 5 minutes. Finally, each cell was resuspended in 1 ml of DEP experimental medium.

3.7.2 Experimental set up

5Vp-p sinusoidal electrical signals were supplied to the DEP device by a function generator (Textronix, USA) to generate non-uniform electric field required for DEP. The experiments were carried out by applying one signal to all four dots even though the device was capable to receive four signals at the same time. For each set of cells (PBMC and monocyte), 15 individual frequencies over a range of 10 kHz to 2 MHz were transmitted via the electrodes. The DEP device was located on a microscope stage (BX51, Olympus, Japan) and the plane of the cells was focused to discern the movement of the cells triggered by the DEP force. The images of the cells were captured by a CCD camera (Dino Capture, USA) which was placed into one of the microscope eyepieces. The experiment was performed three times for each frequency and each sample. The image was captured after 10 seconds of applying the electrical signal. All the images were saved in the dedicated computer for the analysis.

3.7.3 Image analysis

The dielectrophoresis force applied to each cell subpopulation cannot be directly quantified. Hence, to determine dielectrophoresis response of the PBMC and monocytes, the obtained microscopic image from this experiment were analysed. The diagram processing steps of the image summarize in Figure 3.2.