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**INTEGRATED MICROFLUIDICS FOR
LABEL-FREE LEUKOCYTE SORTING AND
ELECTRICAL PHENOTYPING**

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SCHOOL OF MECHANICAL AND AEROSPACE ENGINEERING

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INTEGRATED MICROFLUIDICS FOR LABEL-FREE LEUKOCYTE SORTING AND ELECTRICAL PHENOTYPING

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School of Mechanical and Aerospace Engineering

A thesis submitted to the Nanyang Technological University in partial
fulfilment of the requirements for the degree of Doctor of Philosophy

2019

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

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Supervisor Declaration Statement

I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the research and writing are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

8-May-2019

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King Ho Holden Li

Authorship Attribution Statement

This thesis contains material from 3 papers published in the following peer-reviewed journals and 1 book chapter in which I am listed as an author.

Chapter 2 (Literature review) is published as 1 review paper and 1 book chapter.

1. C. Petchakup, K.H.H. Li and H.W. Hou. Advances in Single Cell Impedance Cytometry for Biomedical Applications. *Micromachines* **8**(3), 87 (2017). DOI: 10.3390/mi8030087. The contributions of the co-authors for the first paper are as follows:

- I prepared the manuscript drafts. The manuscript was revised by K.H.H. Li and K.H.H. Li and H.W. Hou.

2. N. Liu, C. Petchakup, H.M. Tay, K.H.H. Li and H.W. Hou. Spiral Inertial Microfluidics for Cell Separation and Biomedical Application. *Applications of Microfluidic Systems in Biology and Medicine* **5**, 99 (2019). DOI: 10.1007/978-981-13-6229-3_5. The contributions of the co-authors for the book chapter are as follows:

- C. Petchakup wrote 2 sections in the book chapter namely 1) recent advances and 2) conclusions and future outlook.
- N. Liu wrote introduction and working principles sections.
- H.M. Tay wrote applications section.
- H.W. Hou wrote classification of spiral microfluidics section.
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- I performed all the device fabrication, cell separation (except for monocyte subsets) and electrical measurement and conducted most of the flow cytometry analysis and post data analysis.

- H.M. Tay aided in operating flow cytometry analysis and fluorescence imaging.
- W.H. Yeap and S.C. Wong assisted in the isolation monocyte 3 subsets.
- H.W. Hou conducted the data analysis for diabetes samples.
- H.M. Tay, H.W. Hou and I prepared the manuscript drafts. The manuscript was revised by all authors.

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- I performed all the device design, device fabrication, device operation and electrical measurement and conducted most of the flow cytometry analysis and post data analysis.
- H.M. Tay aided in operating flow cytometry analysis, well plate assay and fluorescence imaging.
- I prepared the manuscript drafts. The manuscript was revised by all authors.

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Chayakorn Petchakup

ABSTRACT

Circulating leukocytes (white blood cells) in blood are known to orchestrate various biological processes and become activated during host defence (e.g. infection) or in pathogenesis of major diseases such as cancer, type 2 diabetes mellitus (T2DM) or cardiovascular diseases. Conventionally, fluorescence- and magnetic-activated cell sorting (FACS, MACS) techniques widely used in leukocyte studies require antibodies labelling which is expensive and time consuming. Leukocytes are also prone to activation during sample preparation which advocates the need to develop novel label-free leukocyte sorting and analysis approaches. Microfluidic impedance cytometry is an established single-cell analysis tool based on intrinsic cellular dielectric properties. It is widely used for leukocyte enumeration and differential counting, but its application in leukocyte activation profiling remains unexplored. In this dissertation, two novel microfluidic technologies for label-free leukocyte sorting and electrical profiling towards rapid immune health profiling in type 2 diabetes mellitus are developed.

In the first part of this project, a combinatorial microfluidic strategy for leukocyte phenotyping by enriching target leukocyte subtypes (neutrophils and monocytes) by Dean Flow Fractionation (DFF) prior impedance measurement is proposed. This increases the detection selectivity which is demonstrated for various applications namely monocyte activation, monocyte differentiation and monocyte subtype characterization. We also showed for the first time, that leukocyte impedance characteristics were associated with cardiovascular risk factors (lipid levels and C-reactive protein (CRP)) in patients with T2DM, thus suggesting leukocyte impedance signature as novel surrogate biomarkers for diabetes testing.

In the second part of the project, both cell sorting module and impedance detection module are integrated on a single chip. The integrated “sample in-answer out” platform provides several key advantages including high leukocyte separation efficiency, minimal sample manual handling, and rapid analysis (~5-15 mins). This platform was developed for direct neutrophil isolation and impedance characterization of neutrophil extracellular trap formation (NETosis), a recently discovered key defense mechanism of neutrophils. Our results showed distinct differences in impedance profiles of neutrophils undergoing NETosis and can be further developed to study neutrophil dysfunction in T2DM. Finally, by changing the dimensions of microfluidic design, it is demonstrated that it is possible to perform cancer cell sorting and electrical phenotyping for assessment of metastatic potential.

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TABLE OF CONTENTS

ABSTRACT.....	VII
ACKNOWLEDGEMENTS	IX
TABLE OF CONTENTS	XI
LIST OF FIGURES	XV
LIST OF TABLES	XXII
LIST OF ACRONYMS	XXIII
LIST OF SYMBOLS	XXV
CHAPTER 1 INTRODUCTION.....	1
1.1 Background.....	1
1.2 Motivation.....	4
1.3 Scope of work	5
1.4 Outline of the thesis	7
CHAPTER 2 LITERATURE REVIEW	9
2.1 Microfluidics for cell separation	9
2.1.1 Passive separation technique.....	11
2.1.2 Active separation techniques	26
2.2 Microfluidic impedance cytometry for single cell analysis	34
2.2.1 Dielectrophoresis and electrorotation	38
2.2.2 Impedance cytometry	39
2.3 Integrated microfluidic platform.....	56
2.4 Summary	59
CHAPTER 3 MATERIALS AND METHODS.....	61
3.1 Device design principle.....	61

3.1.1	Combinatorial strategy for leukocyte phenotyping.....	61
3.1.2	Integrated platform for leukocyte sorting and impedance profiling	65
3.2	Device fabrication.....	67
3.3	Leukocyte preparation	69
3.3.1	Leukocyte isolation.....	69
3.3.2	Monocyte treatment	69
3.3.3	Macrophage differentiation from primary monocytes	69
3.3.4	Monocyte subsets sorting.....	70
3.3.5	THP-1 differentiation.....	70
3.3.6	ox-LDL uptake by THP-1 macrophages.....	71
3.3.7	Sample processing for NETosis studies.....	71
3.3.8	Immunofluorescent staining.....	71
3.3.9	Quantification of NETosis using microplate reader	72
3.4	Flow cytometry analysis	72
3.5	Impedance simulation	72
3.5.1	The effect of size variation on impedance response	75
3.5.2	The effect of cell membrane properties variation on impedance response	
	76	
3.5.3	The effect of cytoplasm properties variation	79
3.5.4	The effect of nucleus size variation	80
3.5.5	The effect of different models.....	80
3.6	Impedance measurement setup	82
3.7	Data presentation and statistical analysis.....	83
3.8	Study approval	84
3.9	Summary	85

CHAPTER 4 COMBINATORIAL MICROFLUIDIC STRATEGY FOR LABEL-FREE LEUKOCYTE SORTING AND IMPEDANCE-BASED PROFILING 87

4.1	Background	87
4.1.1	Activation profiling and diabetes profiling	89
4.1.2	Monocyte differentiation characterization	89
4.1.3	Cancer cell identification	90
4.2	Experimental section.....	90
4.2.1	Leukocyte impedance measurement	90
4.3	Results and discussion	91
4.3.1	Impedance-based leukocyte subtypes identification.....	91
4.3.2	Impedance-based leukocyte activation profiling	94
4.3.3	Impedance-property of monocyte differentiation into macrophages	97
4.3.4	Monocyte subset characterization.....	98
4.3.5	Impedance-based immune health profiling in type 2 diabetes mellitus (T2DM).....	100
4.3.6	Monocytes and cancerous monocytic cells comparison	104
4.3.7	ox-LDL uptake by THP-1 macrophages.....	104
4.4	Summary	106

CHAPTER 5 INTEGRATED PLATFORM FOR LEUKOCYTE SORTING AND IMPEDANCE PHENOTYPING107

5.1	Background	107
5.1.1	Formation of neutrophil extracellular traps (NETosis).....	107
5.1.2	Cancer cell phenotyping	109
5.2	Experimental section.....	109
5.2.1	Device operation	109

5.3 Results and discussion	110
5.3.1 Flow rate characterization with different sized particles	110
5.3.2 Characterization of neutrophil sorting from lysed blood and diluted blood 112	
5.3.3 Characterization of monocyte sorting from PBMCs	117
5.3.4 Impedance identification of sorted neutrophils and monocytes	119
5.3.5 NETosis electrical profiling for functional assessment	120
5.3.6 Cancer cell sorting and discrimination	124
5.4 Conclusion	127
CHAPTER 6 CONCLUSIONS AND FUTURE WORKS.....	129
6.1 Conclusions.....	129
6.2 Future works	130
6.2.1 Novel electrode fabrication scheme: self-aligned contact electrodes using liquid metal	130
6.2.2 Reduction of equilibrium positions in the integrated inertial-impedance cytometry platform.....	132
6.2.3 On integrated functionality: post active sorting.....	133
6.2.4 Potential applications of impedance cytometry	133
REFERENCES.....	135

LIST OF FIGURES

- Figure 2.1 Microfiltration-based microfluidics (A) Four types of microstructure used in filtration (Ji et al. 2008) (B) Crossflow filtration device by VanDelinder and Groisman (2006). Ports labeled 1-4 are blood inlet, perfusion inlet, WBC outlet, and RBC outlet, respectively. Channel depths of 25, 9, and 3 μm were indicated by different shades of gray scale (VanDelinder and Groisman 2006). 13
- Figure 2.2 Biomimetic separation (A) Plasma separation device from Zweifach-Fuch law (Yang et al. 2006) (B) Effect of constriction channel: Downstream plasma layer is larger than plasma layer before flowing in constriction region (Faivre et al. 2006)..... 15
- Figure 2.3 Pinched Flow Fractionation (PFF) and Deterministic Lateral Displacement (DLD) (A) PFF-based microfluidic device both original design and asymmetrical branch design (Takagi et al. 2005). (B) DLD device by Huang et al. (2004). 17
- Figure 2.4 Affinity flow fractionation: (a) The two inlets and two outlets device for affinity flow fractionation designed by Bose et al. (2013) (b,c) Schematic of device at inlet and outlet sections. (d) Parallel gold stripes array coated with P-selectin (e) The neutrophil separation using this technique (Bose et al. 2013)... 19
- Figure 2.5 Inertial microfluidics in particle separation (A) Inertial focusing effect in a straight channel (B) High aspect ratio straight channel for bacteria filtration by Mach *et al.* (Mach and Carlo 2010) (C) Microvortices in contraction and expansion channel (top) and microscopic images of particle trapping in expansion section (bottom) taken from Mach et al. (Mach et al. 2011). Arrow head indicates strength of shear-gradient lift force. (D) Spiral inertial microfluidics (E) Our previously developed device for leukocyte separation (Hou et al. 2016). 21
- Figure 2.6 Integrated multiplexing spiral devices: (A) Photo of a multiplexed spiral device by stacking 3 spiral devices together for high throughput CTCs isolation. Adapted with permission from (Khoo et al. 2014), copyright 2014, PLoS; (B) Image of a stacked spiral system for large range particle separation. Reproduced with permission from (Miller et al. 2016a), copyright 2016, AIP Publishing; (C) Schematic illustration of cascaded sample processing using different spiral devices. Reproduced with permission from (Miller et al. 2016b), copyright 2016, Nature; (D) (left) Recirculation spiral microfluidics strategy. (right) Images and plots showing sputum and purified samples using their system (C-sep) and DTT protocol. Adapted with permission from (Ryu et al. 2017), copyright 2017, ACS Publications; 25
- Figure 2.7 Magnetic-based separation (A) Schematic of Han and Frazier (2006) device for magnetic separation: Single cascade paramagnetic separation device Under applied magnetic field, particles with different magnetic properties will align at different lateral positions enabling particle collection. (B) three cascade paramagnetic separation device was introduced to further enhance separation efficiency. Ferromagnetic wires are displayed in gray color. 26
- Figure 2.8 Acoustophoresis-based separation (A) Schematic of Petersson et al. (2007) device. On the top picture shows when different sized particles experience acoustic field generated by an ultrasonic transducer and align into different flow streams. On the bottom picture shows the outlet configuration of the device for

collection of different sized particles. **(B)** SSAW based microdevice by Nam et al. (2011). Focused blood stream experiences surface acoustic wave generated by interdigitated transducer on LiNbO₃, different blood components align to the pressure node or anti-pressure node enabling platelet extraction from blood..... 30

Figure 2.9 Dielectrophoresis-based microfluidics **(A)** Illustration of DEP working principle (Watarai 2013) **(B)** DEP device for platelet separation by liquid electrodes. Hydrodynamically focused mixed samples flows to DEP electrodes region where different particles experience different DEP forces enabling separation of platelet from RBC and WBC. (*Piacentini et al. 2011*)..... 32

Figure 2.10 Micro-fluorescence-activated cell sorting (μ FACs) from Wolff et al. (2003) **(A)** Device schematic **(B)** Scanning Electron Microscope (SEM) image of μ FACs showing 1. Focusing of sample stream (b) by sheath stream (a) 2. Optical interrogation window (c) 3. Reservoir for sorted cells (d). 33

Figure 2.11 **(A)** (Left) Illustration of coplanar electrodes design and impedance signal response when a cell flows through the detection region. (Right) Impedance response at different frequencies carries different information regard the cell. Reproduced with permission from (Gawad et al. 2001), copyright 2001, Royal Society of Chemistry; **(B)** Illustration of parallel electrodes design. Reproduced with permission from (Cheung et al. 2005), copyright 2005, John Wiley and Sons; **(C)** Illustration of constriction channel design. Reproduced with permission from (Chen et al. 2011), copyright 2011, Royal Society of Chemistry; **(D)** Diagram shows the measurement setup. Reproduced with permission from (Sun et al. 2009), copyright 2008, Springer;..... 43

Figure 2.12 **(A)** Illustration of liquid electrode design. Reproduced with permission from (Valero et al. 2010), copyright 2010, Royal Society of Chemistry; **(B)** Illustration of combination approach of conventional coplanar electrode design and liquid electrode design. Reproduced with permission from (Shaker et al. 2014), copyright 2014, Royal Society of Chemistry; 46

Figure 2.13 **(A)** Illustration of constriction channel design with dual frequencies measurement **(B)** Equivalent circuit model and current paths at low and high frequency when constriction channel with cell and without cell. Reproduced with permission from (Zhao et al. 2013a), copyright 2013, Elsevier;..... 48

Figure 2.14 **(A)** Plot of opacity (10MHz/503kHz) versus impedance signal at 503kHz for white blood cells population after addition of CD4 beads. Reproduced with permission from (Holmes and Morgan 2010), copyright 2010, American Chemical Society; **(B)** (Left) Schematic of impedance cytometer with on-chip waveguide (Right) 3-D scatter plot of side scatter impedance and fluorescence for CD4 labelled white blood cells population. Color represents opacity magnitude. Reproduced with permission from (Spencer et al. 2014a), copyright 2014, Royal Society of Chemistry; **(C)** (a) Schematic of sample pretreatment module proposed by van Berkel *et al.* (van Berkel et al. 2011) for whole blood processing prior impedance detection. The design includes two pathways 1. Sample dilution followed by red blood cells and platelets counting 2. Red blood cells lysis and quenching followed by white blood cells differential counting. Reproduced with permission from (van Berkel et al. 2011), copyright 2011, Royal Society of Chemistry; **(D)** Schematic of on-chip sample pretreatment with capture chamber for differential counting of CD4 or CD8. 1. Inlets for loading of whole blood and

reagent solutions (lysing buffer and quenching buffer). 2. Lysing followed by quenching 3. Entrance counting 4. CD4 or CD8 capture chamber 5. Exit counter. Reproduced with permission from (Hassan et al. 2016), copyright 2016, Nature Publishing Group;.....	52
Figure 2.15 (A) Scatter plot of signal amplitude at 10MHz versus 602kHz for 5,6 μ m beads, red blood cells and glutaraldehyde fixed red blood cells at different concentrations. Reproduced with permission from (Cheung et al. 2005), copyright 2005, John Wiley and Sons; (B) Real part of signal amplitude versus imaginary part of signal magnitude of different kinds of red blood cells (ghost, normal, parasite infected). Reproduced with permission from (Küttel et al. 2007), copyright 2007, Elsevier; (C) (Top) Histogram shows the distribution of in-phase amplitude from platelets and red blood cells. (Bottom) Scatter plot of discriminant analysis of non-activated platelets and TRAP activated platelets. Reproduced with permission from (Evander et al. 2013), copyright 2013, Royal Society of Chemistry;	54
Figure 2.16 (A) Scatter plot of opacity (2MHz/500kHz) and diameter (impedance signal at 500kHz) for leukocytes and MCF7 cells. Color represents fluorescence signal. Reproduced with permission from (Spencer et al. 2014b), copyright 2014, AIP Publishing LLC; (B) Scatter plot of $C_{\text{specific membrane}}$ versus $\sigma_{\text{cytoplasm}}$ for A549 (mouse I) and H1299 (mouse IV). Reproduced with permission from (Huang et al. 2015), copyright 2016, Nature Publishing Group;	55
Figure 2.17 Integrated microfluidic platform (A) Integrated platform for circulating tumor cells sorting by Toner <i>et al.</i> (Karabacak <i>et al.</i> 2014) (B) (left) Schematic of integrated spiral device with sequential lateral cavity acoustic transducer. (right) Microstreaming vortices in LCAT channel for particle trapping. Adapted with permission from (Nivedita et al. 2017), copyright 2017, Royal Society of Chemistry; (C) (top) Workflow and image of the integrated spiral device with on-chip RBC lysis. Reproduced with permission from (Ramachandraiah et al. 2017), copyright 2017, Royal Society of Chemistry;	59
Figure 3.1 Device layout for combinatorial strategy for leukocyte phenotyping (A) device layout of DFF sorter (left) and schematic illustration of DFF separation (right) (Hou et al. 2016) (B) device layout of impedance cytometer (left) and schematic illustration of impedance detection.	63
Figure 3.2 Integrated platform for leukocyte subset sorting and impedance phenotyping Layout of the integrate platform (top) Schematic illustrations depicting target cells flow behavior at different stages namely the particle sorting (DFF) (stage 1), flow rate reduction (FRR) (stage 2), and impedance detection (stage 3) (bottom).	65
Figure 3.3 Hydraulic circuit of the integrated platform: Hydraulic resistances of the microfluidic network are designed such that the flow rate is greatly reduced by 40 $\times$ prior impedance detection channel.....	67
Figure 3.4 Impedance simulation model for leukocytes (A) Schematics of equivalent circuit model of a cell inside detection region (left) double shell model for describing leukocytes (right) (B) Equivalent circuit model of single cell impedance measurement (left) and illustration of a single cell in the detection region.....	75
Figure 3.5 The effect of cell size variation on cell current response	76

Figure 3.6 (A) Effect of cell membrane variation on impedance spectra (B) Effect of cell membrane variation on opacity spectra.....	77
Figure 3.7 Effect of cytoplasm variation on opacity spectra	79
Figure 3.8 The effect of nucleus size variation on opacity spectra.....	80
Figure 3.9 Effect of different model on opacity spectra	81
Figure 3.10 Experimental setup: (A) Schematic of experimental setup illustration of microfluidic chips, (B) optical image illustrating single cell flowing through the electrodes in the detection region and (C) Time domain measurement of multiple events from single cells	83
Figure 4.1 Workflow of leukocyte impedance phenotyping strategies starting with sample pre-processing (RBC lysis or Ficoll density centrifugation), DFF leukocyte sorting and impedance profiling	91
Figure 4.2 Impedance profiling of different blood cell samples (A) Impedance profiles (density scatter plot of cell size ($ Z_{LF} $, V) versus opacity) of diluted whole blood (1000 $\times$ dilution), PBMCs, DFF-sorted monocytes, DFF-sorted lymphocytes and DFF-sorted neutrophils. Impedance profile was gated into 3 different zones based on cell size to distinguish cellular debris, platelets and RBCs (zone 1: 0.0004-0.002V), lymphocytes (zone 2: 0.002-0.007V), and monocytes and neutrophils (zone 3: 0.007-0.015V). Due to high density of zone 1 population, the scatter density of zone 1 was calculated separately from zone 2 and zone 3. (B) Frequency distribution of different leukocyte subtypes in each zone for unsorted PBMCs and DFF-sorted leukocytes to determine cell purity.	92
Figure 4.3 Impedance-based activation profiling of enriched monocytes (A) Representative impedance profile of untreated (left), TNF- α treated (center) and LPS treated monocytes (right) with number of cells (n) (B) Average monocyte opacity (left) and cell size (right) for different conditions. Values were taken from the centroid positions of impedance profile for untreated, TNF- α , LPS _{low} (black circle) and LPS _{High} (green circle). Bars were represented as mean $\pm$ s.e.m. *P $\leq$ 0.05, **P $\leq$ 0.01 (C) Flow cytometry analysis of leukocyte composition of PBMCs and DFF-sorted samples (n=4). (D) Flow cytometry analysis of monocyte activation markers CD14 (top) and CD11b (bottom) at different conditions.	94
Figure 4.4 The effect of LPS concentration and duration variation on impedance profile of sorted leukocytes (A) Impedance profiles of different conditions namely control sample (healthy monocytes), sample treated with low concentration of LPS for 4 hours, sample treated with LPS for 4 hours and sample treated with LPS for 8 hours; Blue and red boundary are used to highlight LPS _{high} and LPS _{low} respectively. (B) Flow cytometry analysis of leukocyte composition of control and LPS treated monocytes at different durations and doses. (n=2)	96
Figure 4.5 Characterization of monocyte differentiation into macrophages and monocyte subsets characterization (A) 20 $\times$ Brightfield images of seeded monocytes (day 0), and macrophages after 4 days 7 days of differentiation with M-CSF. Scale bar = 20 μ m (B) Impedance profile of monocytes, monocytes undergoing differentiation at day 4, and fully-differentiated macrophages at day 7 (M Φ). (C) Average monocyte size (bottom) and opacity (top) at different differentiation stages. Bars were represented as mean $\pm$ s.e.m. *P < 0.05, **P < 0.01	97

- Figure 4.6 Characterization of monocyte subsets characterization (A) Representative impedance profiles of classical (left), intermediate (center) and non-classical monocytes (right) (B) Raw data for opacity and cell size before normalization from 4 different subjects (C) Normalized opacity (left) and size (right) for monocyte subtypes from 4 different subjects. The normalization was acquired by dividing opacity of each subset by the average value of every subsets (same subject). Paired t-test was used to determine the statistical significance of the difference between two sets of data. *P <0.05, **P <0.01. (D) Impedance profiles of 1. unstained monocytes 2. stained monocytes (before FACs sorting) 99
- Figure 4.7 Impedance-based immune health profiling in type 2 diabetes mellitus (T2DM) (A) Measurement of cell size and opacity of monocytes and neutrophils in T2DM patients. (B) Strong correlation of monocyte and neutrophil opacity in T2DM patients. (C) Scatter plot of monocyte impedance phenotype in T2DM patients (n = 8). Blue shaded box indicates impedance profile distribution of non age-matched healthy monocytes (n=4) and is plotted based on geometrical mean ± 2 S.D. Blue dot represents an age-matched healthy subject which resides within the healthy zone. (D) Representative brightfield and immunofluorescence images of T2DM monocytes with the presence of monocyte-platelet aggregates (MPAs) after DFF. 101
- Figure 4.8 Correlation of monocyte and neutrophil impedance parameters with (A) Non-HDL cholesterol, (B) LDL Cholesterol, (C) LnRHI, (D) Hyperlipidemia and correlation of neutrophil impedance parameters with (E) C-reactive protein (CRP) and (F) Hyperlipidemia 102
- Figure 4.9 Correlation of monocyte and neutrophil impedance parameters with (A) Correlations of monocyte size with neutrophil size, correlations between average opacity and cell size from monocyte (B) and neutrophil (C) and correlations of average monocyte size with other clinical relevant parameters such as (D) total cholesterol and (E) triglyceride 103
- Figure 4.10 Other applications (A) Discrimination of cancer cells (B) Characterization of ox-LDL uptake by THP-1 macrophages; Black dotted line represents threshold for LDL uptake determination. (Right) Microscopic images of control (top) and LDL sample (bottom). Red color represents dyed LDL internalized by the cell. 105
- Figure 5.1 Neutrophil Extracellular Traps (NETs) formation (Papayannopoulos 2017) 108
- Figure 5.2 Workflow of integrated platform for leukocyte subset sorting and impedance phenotyping 110
- Figure 5.3 Flow rate characterization of 10 μm beads at different device stages: (A) Composite fluorescence images and (B) intensity profiles of particle sorting, 1st FRR and 2nd FRR prior impedance detection at different sample flow rates ranging from 130 - 170 $\mu\text{L}/\text{min}$. Scale bar = 100 μm 111
- Figure 5.4 Flow rate characterization of 5 and 7 μm beads at the sorting stage: Composite fluorescence images of particle sorting at different sample flow rates ranging from 130 - 170 $\mu\text{L}/\text{min}$. Scale bar = 100 μm 112
- Figure 5.5 Performance evaluation of the integrated platform with bead mixture (A) High-speed images for different sized bead mixture (5, 7, 10 μm). Scale bar = 100

μm (B) Flow cytometric analysis of sorting efficiency (left), enrichment (center) and concentration ratio (right)	113
Figure 5.6 Performance evaluation of neutrophil sorting from lysed blood (A) High-speed images showing cells focusing at different flow rates and different parts of the device. Scale bar = 100 μm (B) Flow cytometric analysis of neutrophil sorting efficiency, (C) platelet depletion, (D) enrichment and (E) concentration ratio	115
Figure 5.7 Performance evaluation of neutrophil sorting from diluted whole blood (500×) (A) High-speed images showing cells focusing at different flow rates and different parts of the device. Scale bar = 100 μm (B) Flow cytometric analysis of neutrophil sorting efficiency, (C) RBC depletion, (D) platelet depletion, (E) neutrophil enrichment and (F) concentration ratio.....	116
Figure 5.8 Performance evaluation of monocyte sorting from PBMCs (A) High-speed images showing cells focusing at different flow rates and different parts of the device. Scale bar = 100 μm (B) Flow cytometric analysis of monocyte sorting efficiency, (C) platelet depletion, (D) enrichment and (E) concentration ratio	118
Figure 5.9 Impedance profiling of different leukocytes from different blood samples The impedance profiles were represented as a 2D scatter distribution plot based on cell size ($ Z_{LF} $, impedance magnitude at 0.3MHz) and opacity ($ Z_{HF} / Z_{LF} $, ratio of impedance magnitude at 1.72MHz to 0.3MHz) (A) impedance profile of sorted neutrophils highlighted in green and spiked 10 μm beads highlighted in red (left) and illustration of particle positions inside detection channel (right), representative impedance profiles of (B) sorted neutrophils from lysed blood, (C) sorted neutrophils from diluted blood (500×) and (D) sorted monocytes from PBMCs. For every plot, the cell opacity was normalized by the average opacity of reference beads.	120
Figure 5.10 NETosis profiling using impedance cytometry. (A) Overlaid fluorescent (Hoechst and SYTOX Green) and brightfield images showing NETosis progression (using CaI) over the course of 2 hour. Red dotted lines highlight cell boundary determined from brightfield images. Scalebar = 10 μm (B) Fluorescent intensity of SYTOX green for healthy neutrophils and glucose-treated neutrophils undergoing NETosis. Data are presented as mean ± s.e.m (n = 4). *P ≤ 0.05. (C) Representative impedance profiling of neutrophils sorted from lysed blood undergoing NETosis at different time points. Dotted lines indicate means of each cluster. (D) Average relative neutrophil cell size (left) and opacity (right) with respect to unstimulated neutrophil (0 min) for control and glucose-treated data	123
Figure 5.11 Hydraulic circuit of the integrated platform for cancer cell applications: Hydraulic resistances of the microfluidic network are designed such that the flow rate is greatly reduced by 49.6× prior impedance detection channel.	124
Figure 5.12 Flow rate characterization of 15 μm beads at different device stages: Composite fluorescence images of particle sorting at different sample flow rates ranging from 150 - 200μL/min. Scale bar = 100 μm	125
Figure 5.13 High-speed images of cells focusing at different flow rates and different parts of the device (A) MDA-MB 231 (B) MCF-7	126
Figure 5.14 Characterization of cancer cells Impedance profiles of MCF-7 (left) and MDA-MB 231 (right).....	127

Figure 6.1 (A) Schematic of microfluidic device with self-aligned liquid metal electrode. Green box provides close-up images of detection region. (B) Preliminary studies using EGaIn	132
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LIST OF TABLES

Table 2.1 Comparisons between different microfluidic cell separation techniques	34
Table 2.2 Comparisons between different impedance cytometers design	49
Table 2.3 List of developed impedance cytometry applications based on cell type. WBCs: white blood cells; RBCs: red blood cells; TRAP: thrombin receptor activating peptide; PDMS: polydimethylsiloxane.....	49
Table 3.1 Simulation parameters	81
Table 4.1 Clinical demographics of T2DM patients.....	104

LIST OF ACRONYMS

WBC	White blood cell
RBC	Red blood cell
FACs	Fluorescence-activated cell sorting
MACs	Magnetic-activated cell sorting
LOC	Lab on a chip
DLD	deterministic lateral displacement
CTC	Circulating tumor cell
DFF	Dean flow fractionation
T2DM	Type 2 diabetes mellitus
PBMC	Peripheral blood mononuclear cell
TNF- α	Tumor necrosis factor alpha
LPS	Lipopolysaccharide
CRP	C-reactive protein
PCR	Polymerase chain reaction
PDMS	Polydimethylsiloxane
PFF	Pinched flow fractionation
AFF	Affinity flow fractionation
HCT	Hematocrit
IgG1	Immunoglobulin G1
BAW	Bulk acoustic wave
SSAW	Standing surface acoustic wave
DEP	Dielectrophoresis
DC	Direct current

AC	Alternative current
BioMEMs	Biomedical microelectromechanical systems
FRR	Flow rate reduction
M-CSF	Macrophage colony-stimulating factor
PBS	Phosphate buffered saline
BSA	Bovine serum albumin
LDL	Low density lipoprotein
FITC	Fluorescein isothiocyanate
PE	Phycoerythrin
APC	Allophycocyanin
DBSCAN	Density-based spatial clustering of applications with noise
NET	Neutrophil extracellular traps

LIST OF SYMBOLS

Z = Impedance

Subscripts

- LF – low frequency
- HF – high frequency
- Mix – mixture
- Med – medium

V = Voltage

I = Current

ε_{eff}^* = Effective complex permittivity

ε^* = Complex permittivity

Subscripts

- out – outer
- in - inner
- mix – mixture
- nucleus – nucleus
- np – nucleoplasm
- ne – nucleus envelop
- cn – cytoplasmic contain
- cp – cytoplasm
- cell – cell
- mem – cell membrane

σ = Conductivity (Subscripts are similar to complex permittivity)

ε = Permittivity (Subscripts are similar to complex permittivity)

φ = Volume fraction of inner component to outer component

Subscripts

- 1 - the ratio of volume of nucleus to volume of cytoplasm
- 2 - the ratio of volume of nucleoplasm to volume of nucleus
- mc - the ratio of volume of cytoplasm to volume of cell

Φ = Volume fraction which is the ratio of volume of cell (sphere) to volume of detection channel

ω = Angular velocity

l = Channel width

w = Channel width

h = Channel height

F_L = Net lift force

$U_{\max}$ = Maximum fluid velocity

D_h = Hydraulic diameter

C_L = Lift coefficient

a_p = Particle diameter

F_D = Dean drag force

De = Dean number

Re = Reynold number

R = Radius of curvature

U_L = Lateral migration velocity

L_1 = Channel length

L_D = Migration length

U_{Dean} = Dean velocity

U_f	=	Average fluid velocity
ΔP	=	Pressure difference
Q	=	Volumetric flow rate
R_h	=	Hydraulic resistance
r_h	=	Hydraulic radius of the channel
A	=	Cross sectional area
P	=	Perimeter of microchannel
ε_0	=	Permittivity of free space
d	=	Thickness of cell membrane
R_{ne}	=	Nucleus size

CHAPTER 1 INTRODUCTION

1.1 Background

Blood is one of the most essential fluids circulating in our body because blood contains various cellular components responsible for different indispensable functions. Among cellular components in blood, white blood cells (WBCs) or leukocytes constitute approximately 1% of all blood cells and they involve in many immune functions as well as pathogenesis of diseases thus understanding their cellular heterogeneity and biological significance can lead to development of novel disease detections or therapeutic interventions (1997; 2007; 1991; Kantari et al. 2008). However, it is non-trivial task to study leukocytes because of blood complexity as other high-density components, such as red blood cells (RBCs), might hinder the presence of leukocytes adversely affecting subsequent analysis. In addition, interplay between components might affect how the target component behaves and may result in undesired effects (Chen and Cui 2009; Toner and Irimia 2005). Hence, an efficient leukocyte separation is of importance prior any leukocyte studies.

To separate leukocyte subset for study, several conventional techniques include microfiltration, density gradient centrifugation, fluorescence-activated cell sorting (FACs) and magnetic-activated cell sorting (MACS) are possible. Microfiltration utilizes arrays of micropores/sieves to filter out smaller particles from larger particles in suspension. The common problems of this technique are clogging deteriorating the device performance. Density gradient centrifugation exploits the density differences of cellular components to separate different cell types. This technique is expensive due to equipment and additional mediums (e.g. Ficoll, Percoll). Furthermore, it also requires skilled expertise to perform cell extraction which is laborious and time-consuming.

FACs is commonly employed in leukocyte studies. The cells were first labelled with specific fluorescent antibodies prior analysis, followed by sorting based on cell optical or fluorescence characteristics. Similar to FACs, MACs requires the sample to be bound with antibody-coupled magnetic beads then the labelled cells can be acquired or discarded under strong magnetic field. Although both MACs and FACs can provide various advantages such as high throughput and high separation efficiency, the use of antibodies labelling has several drawbacks such as expensive, time consuming and prone to causing adverse effects to the cells (Jensen 2012). Hence, it is imperative to develop a novel sorting technology capable of providing leukocyte subsets in native state which can potentially facilitate leukocyte research.

Since the emergence of microfluidic technology, this enables scientists to fabricate a miniaturized device that can be used to manipulate small amount of fluid (Whitesides 2006; Yager et al. 2006). Its utilization is found to be useful for blood analysis applications because it can potentially reduce the need of traditional technologies such as the need of expertise, laborious process, reagent and sample consumption and expensive equipment. In addition, a key advantage over traditional method is that various essential elements such as sample preparation, separation, concentration and single analysis can be integrated together in a single chip so called Lab-On-a-Chip (LOC).

Typically, the analysis of cell's function and properties can be broadly classified into two groups which are population-averaged analysis and single-cell analysis. For population-averaged analysis, average information of cells' function or properties was acquired from highly heterogeneous sample such as blood (Altschuler and Wu). Thus, averaging the information can possibly lead to obscure information about rare

population. Among single cell characterization techniques, impedance cytometry is an attractive alternative for label-free single cell characterization based on size and intrinsic electrical properties of cellular components such as cell membrane and cytoplasm (Chen et al. 2015; Cheung et al. 2010; Petchakup et al. 2017; Sun and Morgan 2010). Similar to FACs, it also possesses compelling features such as high throughput and multiparametric analysis. This technique has been widely exploited for cell type identification (Hassan et al. 2017; Simon et al. 2016; Spencer et al. 2014a), activation profiling (Evander et al. 2013; Rollo et al. 2017) as well as discrimination of rare cell or parasitic cells (Du et al. 2013; Spencer et al. 2014b). However, there is still a lack of its utilization for leukocyte phenotyping to observe how diseases or chemical stimulus alter physiological properties of cells which would be critical for identification of a novel surrogate biomarker for disease detection or stratification. It has several drawbacks namely low flow rate operation and unable to handle complex samples without pre-processing (Chen et al. 2015; Petchakup et al. 2017; Sun and Morgan 2010). Although this technique can be used to identify all major blood components, it will take considerable amount of time to acquire statistical meaningful data for leukocytes as they are minorities in blood. Moreover, the presence of abundant smaller components (RBCs and platelets) can co-flow with leukocytes through the detection region simultaneously, thus causing error in impedance readout and complicating signal analysis. Hence, there exists a critical need to develop label-free microfluidic integrated platform for leukocyte sorting and detection with minimal perturbation and manipulation to facilitate leukocyte studies in their native state. Moreover, such a platform can provide automation and assay robustness with minimal user sample manipulation for point-of-care testing.

In the past decade, there have been several integrated or hybrid microfluidic platforms engineered by many research groups (Hassan et al. 2017; Hassan et al. 2015;

Karabacak et al. 2014; Kim and Soh 2009; Mizuno et al. 2013; Nagrath et al. 2007; Sheng et al. 2015; Sheng et al. 2017; van Berkel et al. 2011; Zhang et al. 2014). However, most of these integrated platforms were focused on enhancement of a single function rather than creating a multifunctionalities LOC system; For example, Toner et al. incorporated 3 different microfluidic strategies (deterministic lateral displacement (DLD), inertial focusing and magnetophoresis) to achieve isolation of circulating tumor cells (CTCs). The analysis of sorted CTCs needs to be carried out downstream or on different platform separately (Karabacak et al. 2014). To facilitate the practicality of LOCs, it is critical that the developed platform needs to minimize human handling and reduce total analysis/testing time by incorporating on-chip detection/analysis.

In this dissertation, the realization of integrated platform combining leukocyte subset sorting and on-chip single cell impedance measurement is reported. It is demonstrated with applications of impedance cytometry in leukocyte phenotyping namely monocyte activation, monocyte differentiation and diabetes testing. Next, leukocyte subset sorting based on Dean flow fractionation (DFF) is realized with impedance cytometry on a single platform and the potential applications for the developed platform such as neutrophil functional profiling and cancer cell detection are demonstrated.

1.2 Motivation

As pointed out earlier, the earlier demonstrations of impedance cytometry focused on cell identification and there is a critical lack in applying impedance cytometry for study leukocytes upon activation or operating certain immune function which are of clinical importance for immunology research as well as development of point-of-care

testing. Therefore, it is necessary to explore the feasibility of impedance cytometry as a tool for activation and functional studies.

Another issue with impedance cytometry is unable to handle complex samples without pre-processing. By coupling cell separation with impedance cytometry, it can provide a minimal perturbation and manipulation to facilitate leukocyte studies in their native state. Hence, platform or strategy is suitable for point-of-care applications.

1.3 Scope of work

In the first part, the use of impedance cytometry for various aspects of leukocyte phenotyping is investigated. The impedance cytometer will be designed and developed for carry out experiments. In this part, monocytes are the primary interest. Monocytes represent a highly heterogeneous leukocyte population with the ability to differentiate into macrophages. Both monocytes and macrophages play a major role in the pathogenesis of diabetes related complications (Gordon and Taylor 2005; Sprangers et al. 2016). Thus, it is of clinical importance to develop a novel technique for characterizing these cells to elucidate their biological significance which can potentially pave the way for clinical diagnostics as well as immunology research. In this work, several aspects of monocytes are studied namely activation profiling, monocyte differentiation into macrophage, subtype characterization and monocyte profiling in type 2 diabetes mellitus (T2DM). Overall, all these applications show the potential of impedance cytometry to be used in immunology research or developed into point-of-care testing.

After demonstrating the use of impedance cytometry in leukocyte phenotyping, a inertial leukocyte sorter based on previously developed device in our group (Hou et al. 2016) is integrated with an impedance cytometer on a single platform in a continuous,

rapid and label-free fashion. The major challenge in combining DFF and impedance cytometry is a flow rate disparity between both functionalities as impedance cytometry typically operates at low flow rate ($<50\mu\text{L}/\text{min}$) while DFF operates at high flow rate ($>1\text{mL}/\text{min}$). To bridge this gap, double staged flow rate reduction was integrated to reduce output flow rate of DFF by ~ 6 prior impedance detection. The developed platform will be characterized at different flow rates to observe focusing behaviors of different sized particles representing leukocytes at different section of the device and evaluate sorting efficiency. The sorting efficiency of target cells (neutrophils and monocytes) is demonstrated with different kinds of common biological samples such as diluted blood, lysed blood and peripheral blood mononuclear cells (PBMCs) sample. As a demonstration of applications, the function of neutrophils will be studied. Neutrophils are the most abundant leukocyte in circulation and also provide protection against foreign bodies. Among neutrophil means of defending, NETosis is a novel way of neutrophils to resolve inflammation by releasing neutrophil extracellular traps to capture and kill microbes. During the course of NETosis, neutrophils will undergo various morphological changes. This makes it interesting to see if impedance cytometry can electrically characterize NETosis for label-free functional characterization of neutrophils. On top of that, the integrated platform for cancer cell profiling will be demonstrated by varying a height channel. Characterization and phenotyping of circulating tumor cells are of essence in understanding disease progression as well as developing novel therapeutic approaches. Hence, there is a critical need for the development of rapid and label-free integrated platform for cancer cell sorting and phenotyping towards cancer cell bedside monitoring.

1.4 Outline of the thesis

This thesis consists of five chapters including this introductory chapter.

Chapter 2 will provide a review of relevant literatures. This chapter starts with describing cellular components present in blood. Then we continue with conventional blood separation techniques followed by blood separation on microfluidics which can be categorized into active and passive separation. Next, we discuss single cell characterization techniques. Lastly, we discuss about integrated microfluidic platforms that combine multiple separation or analysis techniques on single chip.

Chapter 3 will cover the materials and methods that have been used for conducting different experiments.

Chapter 4 will present the combinatorial strategies of leukocyte subset sorting using dean flow fractionation (DFF) and single cell electrical profiling using impedance cytometry. In this chapter, the detection principle of impedance cytometry is covered, followed by impedance simulation of particles with different internal complexity namely particle without shell, particle with single shell and with double shell based on Maxwell mixture theory (MMT). Identification of DFF-sorted leukocyte subsets is demonstrated. In addition, various applications of developed strategy namely monocyte activation profiling with different inflammatory stimulus (TNF- α and LPS) are shown, followed by characterization of monocyte differentiation into macrophages as well as monocyte subset identification. As proof-of-concept for diabetes testing, we show quantification of neutrophil and monocyte impedance characteristics and cardiovascular risk factors including lipid levels, C-reactive protein (CRP) and vascular functions (LnRHI) are studied.

Chapter 5 will report on the multi-staged integrated microfluidic platform for direct label-free leukocyte sorting and impedance profiling. The design principle of the integrated platform using equivalent hydraulic circuit analysis is shown. Then flow rate characterization of the platform at different stages using different sized beads (5,7,10 and 15 μ m) are studied. To demonstrate the applications of developed platform, characterization of NETosis which is a key defense mechanism of neutrophils was demonstrated. By changing the dimension of the integrated platform, sorting of cancer cells can be achieved and also the results suggest the feasibility of a single step cancer cell sorting and electrical phenotyping for assessment of metastatic potential.

Lastly, **Chapter 6** will conclude this thesis and present future directions of the presented platform.

CHAPTER 2 LITERATURE REVIEW

2.1 Microfluidics for cell separation

In conventional clinical setup, several common separation techniques are centrifugation, microfiltration, MACs and FACs. Centrifugation is one of the standard techniques used for blood separation. This technique exploits the disparity of buoyant density of different cell types. By centrifugation of blood sample, the centrifugal force causes particles suspended in the blood to sediment into many different layers depending on their density and cellular components can be manually retrieved. This technique is regarded as time consuming and expensive (equipment and reagent) and is unable to separate two components with close density or size. Microfiltration utilizes arrays of micropores/sieves to filter out smaller particles from larger particles in suspension. The common problems of this technique are clogging which deteriorates the device performance. The utilizations of membrane filtration are usually for blood dialyzer, blood transfusion and therapeutic apheresis which is a process that separate undesired components from whole blood and then the rest are returned to the patient (Ward 2011). MACs is a technique that based on immunoreactions of the label element and target cell's surface. Target cells in suspension are labelled by binding specifically with magnetic beads coated with antibodies or other relevant ligands. Magnetically tagged target cells can be captured under strong magnetic field for positive or negative cell selection. Like MACs, FACs is a technique that requires labelling based on immunoreactions. Instead of cell labelling with magnetic particles, the target cells are labelled with fluorophore-conjugated antibodies or other relevant ligands. Once tagged, the target cell will express a different fluorescent property. The suspension is perfused into the flow cytometry machine which focus suspended cells into a single stream for interrogation and separation based on optical and fluorescence properties. It is worth

mentioning that this technique can also provide optical and fluorescence profile of suspended cells/particles (flow cytometric analysis). Although both FACs and MACs are powerful techniques owing to highly specific selection of target cells, they require antibody labelling which is expensive, time consuming and laborious.

Aforementioned methods are commonly used techniques in healthcare or immunology research. Although their efficiency is very high, it should be noted that these traditional methods come with various disadvantages. First these techniques require trained personnel to operate and handle the sample which is prone to cell perturbation. Second, there is no continuity of the blood separation process and subsequent process. For example, after blood separation, the sample has to be handled manually for subsequent analysis; the sample is prone to unexpected effect such as contamination. The amount of sample and reagent used is in large quantity and it also takes rather long processing time. Moreover, for the MACs and FACs, some labelled elements need to be added to the sample. Finally, these machines are too large and not portable which means they cannot be easily relocated to perform on site testing or point of care testing.

To overcome aforementioned issues with conventional cell separation, a variety of Microfluidics for cell separation are developed and can be broadly categorised as active and passive techniques. Active techniques rely on external force fields (electric field, magnetic field and acoustic field) to enable a separation. Typically, these techniques provide low flow rate operation since cells are required to expose to an external force field for certain duration to enable the separation. Some techniques also have specific medium requirements; For example, negative dielectrophoresis scheme needs low conductivity medium; however, it may affect cell viability (Puttaswamy et al. 2010). Additional complicate fabrication process such as sputtering is needed to define

electrodes for external force generation. Despite the disadvantages, this technique offers operational flexibility as a variety of adjustable parameters (excitation waveform, voltage) can be varied on the fly to achieve a separation without a fabrication of new design. Contrary to active techniques, passive techniques do not require any external force field to enable separation. The separation can be done by exploiting channel geometry and inherent hydrodynamic forces present in the microchannel.

2.1.1 Passive separation technique

2.1.1.1 *Microstructure-based separation*

Similar to a conventional counterpart, there were several attempts to miniaturize the microstructure-based filtration into a chip. There are mainly four designs of such microstructures, namely pillar, weir, membrane and crossflow. Four designs of microstructure are shown in Figure 2.1A. Wilding et al. (1998) designed Silicon-based microdevice that has weir and pillar microfilters and was capable of separating white blood cells from whole blood as sample preparation prior Polymerase chain reaction (PCR) analysis. Homsy et al. (2012) designed the membrane-based microfluidic chip for separating plasma from undiluted whole blood and also measured the adsorption of interleukins cells. In this paper, commercially available Vivid membrane from Pall Corporation is used. Vivid membrane is fabricated from asymmetric polysulfone and it also exhibits low non-specific binding of common diagnostic biomarkers. According to the company data sheet, this membrane is capable of recovering plasma from whole blood sample by at least 80%.

Aforementioned techniques are called dead-end filtration in which the flow of the suspension is perpendicular to the microfiltration structure. Hence, these designs suffer from clogging in which large components are stuck at the opening of pores blocking the flow of fluid leading to efficiency reduction.

To overcome the blood clogging issue, another design of microstructure-based separation called crossflow filtration was first introduced by Brody et al. (1995). In this design, the flow of suspension is parallel to the microstructure or the pore opening so that the larger particles are not stuck on the filtration structures, but they are continuously flushed away. Sethu et al. (2006) reported a crossflow microfluidic device for WBCs separation. In this work, the micro sieves were designed to trap WBCs in the main channel and flowed to the WBCs outlet while RBCs can pass through micro sieves to the side and continue to their outlets. At the optimum flow rate, the device was achieved the removal of 50% of RBCs in the main channel along with 97% retention of the inlet WBCs in the main channel. VanDelinder and Groisman (2006) reported a PDMS microfluidic device used to separate WBCs from whole blood sample using perfusion in continuous cross-flow. The device shown in Figure 2.1B composed of the main channel and arrays of lateral side channels (3 μm in height) perpendicularly aligned to both side of the main channel. The narrow channel was 3 μm specifically designed to let only RBCs through. By using sheath/perfusion stream to pinch a sample stream, the small particles (RBCs) were pinched against the side channels and migrated from sample stream to the perfusion and flow to the side channel whereas WBCs cannot flow into the side channels and were washed away to the main channel outlet. As a result, they achieved the WBCs retention of 98% and the ratio of WBCs to RBCs of 2.4. Ji et al. (2008) evaluated the performance of WBCs separation of all the four main designs of microfilter namely pillar, weir, membrane and crossflow. The designed device is shown in Figure 2.1B. Every design used 3.5 μm width microchannels/pores to trap WBCs. They found that, the crossflow design yielded the highest efficiency in capturing the WBCs (70-95%) and passing RBCs (60-95%), followed by pillar design, membrane design and weir design. Recently, Li et al. (2014) developed a continuous-

flow microfluidic cell sorting chip based on crossflow filtration for WBCs separation from unprocessed whole blood. In this work, a filter element used in this work was high porosity Polydimethylsiloxane (PDMS) membrane and also a sheath fluid was used to focus blood sample into underlying membrane structure. As a consequence of focusing, components larger than pore size of the membrane migrated into the sheath fluid stream and proceeded to the top outlet while the smaller components flowed through the membrane and continuously flowed to the bottom outlet. The device can separate $27.4 \pm 4.9\%$ of WBCs from unprocessed blood with $93.5 \pm 0.5\%$ purity.

Overall, this technique features label-free and ease of operation. However, the main issue of this technique is clogging which deteriorates separation efficiency. Another issue is derived from deformability of cellular components as they may slip through the microstructure decreasing target component's purity. Despite all the disadvantages, there are some researches using this technique recently (Li et al. 2014; Wang et al. 2012; Xia et al. 2016) because it is simple and can be easily fabricated. By using well designed geometry of microstructure, microstructure-based separation is

Figure 2.1 Microfiltration-based microfluidics (A) Four types of microstructure used in filtration (Ji et al. 2008) (B) Crossflow filtration device by VanDelinder and Groisman (2006). Ports labeled 1-4 are blood inlet, perfusion inlet, WBC outlet, and RBC outlet, respectively. Channel depths of 25, 9, and 3 μm were indicated by different shades of gray scale (VanDelinder and Groisman 2006).

capable of separating both plasma and cellular components and the high efficiency separation and less clogging issue can be achieved from this technique.

2.1.1.2 Biomimetic separation

Hydrodynamic separation exploits phenomena of blood flowing in microchannel and the laminar flow profile of fluid flow in microchannel to separate blood components. The first part of this section is dedicated to describing related phenomena.

While blood is flowing in the microchannel, suspended RBCs tend to migrate towards the center of microchannel forming cell dense region and lateral plasma region adjacent to the channel wall. This region is called cell-free layer. High shear zones along the wall and deformability of cells promote this phenomenon. Many factors determine the thickness of cell-free layer such as hematocrit, deformability, channel diameter, cell aggregation and flow rate. Since cells dense region is moving faster than the average velocity of the blood flow, this leads to another phenomenon called Fahraeus effects which is the decrement of tube hematocrit of blood as the diameter of channel decreases. Another effect resulting from Fahraeus effects is called Fahraeus-Lindqvist effects. Fahraeus-Lindqvist effect states that the decrease of tube hematocrit leads to the decrease in apparent viscosity. Zweifach-Fung bifurcation law describe cells moving at bifurcation channel where cells encounter anisometric flow rate distribution. Cells tend to move to the branching channel with a higher flow rate instead of moving in the channel with lower flow rate. This causes the channel with lower flow rate to have no or less number of cells. Yang et al. (2006) applied Zweifach-Fung bifurcation law to extract plasma from sheep blood of hematocrit 45%. The design of their device is depicted in Figure 2.2A. The width of primary channel is 15 μm and width of lateral plasma extracting channels is 9.6 μm . As a result, the device was able to extract 15-25% of plasma with 100% separation efficiency.

Fahraeus effect can be enhanced by constriction-expansion region as firstly shown by Faivre et al. (2006). In their design, the cell-free region right after constriction region was increased enabling them to place branching channel parallel to the wall to collect plasma easier. The effect of constriction channel is depicted in Figure 2.2D. Moreover, they also found that increasing length of constriction region could also further enhance cell-free region. Later, many researchers imported this concept in their works and managed to improve separation efficiency in many ways such as design new geometry for collection channel (Sollier et al. 2010), installing two device in series (Marchalot et al. 2014) and utilizing an inertial focusing (Lee et al. 2011).

2.1.1.3 Pinched Flow Fractionation(PFF)

Pinched Flow Fractionation is another technique that utilizes the laminar flow profile in microchannel and a proper channel design to separate suspended particles. The concept of this technique was firstly proposed by Yamada et al. (2004). This technique requires sheath flow to pinch the sample suspension creating a pinched segment where the particles are aligned closely to a sidewall (Figure 2.3A (top)). When the particles flow out of the pinched area into the larger area, the particles will migrate into different streamline according on their size and centroid. The particles will continuously follow their streamlines to collection outlets. In this paper, they examined

Figure 2.2 Biomimetic separation (A) Plasma separation device from Zweifach-Fuch law (Yang et al. 2006) (B) Effect of constriction channel: Downstream plasma layer is larger than plasma layer before flowing in constriction region (Faivre et al. 2006)

several factors that can affect separation performance. First factor was the ratio of flow rates from liquid containing particle and liquid without particle. This factor also determined the width of pinched segment which affected the sidewall alignment of particles. Another factor was the microchannel geometries which were the width of the pinched segment and the boundary angle between the pinched area and broader area. Later, Takagi et al. (2005) from the same group, extended this method by designing asymmetrically arranged multiple branches (Figure 2.3A (bot)). The asymmetrical branch was designed to have low flow resistance because of its shorter length and broader area than other branches which led to asymmetrically distributed flow to each branch. This approach was also used to separate RBCs from diluted whole blood. As a result, approximately 80% of RBCs could be collected from one branch while the rest could be collected in another branch.

The advantage of this technique is label-free process in which no sample pretreatment is required. The clogging can occur if the width of the designed pinched area is too small. Flow rate tuning is needed for proper separation of different particles.

2.1.1.4 Deterministic Lateral Displacement (DLD)

Huang et al. (2004) proposed a new way to separate particles suspended in liquid called Deterministic Lateral Displacement (Figure 2.3B). DLD utilizes asymmetric bifurcation of laminar flow around obstacles to separate particle based on its size. Multiple horizontal rows of obstacles were introduced within the microchannel. In each array of obstacles, it was laterally shifted from the previous row by some distance. The asymmetric bifurcation of laminar flow occurring around obstacles allowed particle to choose its path. Smaller particles tended to flow in a Zigzag manner while larger one preferred to flow in straight line. Zheng et al. (2005) developed a micro device that utilized this technique to separate WBCs and RBCs. The result showed that the WBCs

separation efficiency from diluted whole blood was about 91%. With the fluorescent labelled WBCs, they also observed that RBCs were separated from WBCs. The author also suggested that coating inner wall of microchannel is needed to avoid cell adhesion to the wall. Huang et al. (2008) designed a microfluidic device based on deterministic lateral displacement combined with subsequent magnetic-based separation for isolation of nucleated RBCs from blood of pregnant women. Nucleated RBCs are RBCs containing both Hemoglobin and nucleus. This work demonstrated two step of separation process. The first step was to separate cells based on size and deformation by utilizing deterministic lateral displacement. Because RBCs were smaller than nucleated RBCs and WBCs, RBCs flowed in a Zigzag manner to the waste outlet while nucleated RBCs and WBCs flowed straight to the next part of a chip. As a result, 99% of RBCs were eliminated. In the second step, nucleated RBCs were separated from WBCs by their intrinsic paramagnetic property. When these cells passed through a magnetic column, nucleated RBCs were retained. They achieved 99% of WBC depletion.

Figure 2.3 Pinched Flow Fractionation (PFF) and Deterministic Lateral Displacement (DLD) (A) PFF-based microfluidic device both original design and asymmetrical branch design (Takagi et al. 2005). (B) DLD device by Huang et al. (2004).

The advantages of this technique are similar to pinched flow fractionation. The clogging problem is the primary concern of this technique because of the use of array of obstacles. If the width between each obstacle is not properly designed, suspended particles can easily clog the channel.

2.1.1.5 Affinity Flow Fractionation (AFF)

Bose et al. (2013) introduced Affinity Flow Fractionation method which utilizes a weak-affinity molecular interaction to assist in separation. In this work, they developed the microfluidic device for neutrophil separation from whole blood. To enable the separation, P-selectin was used to loosely bind to neutrophils; P-selectin protein functions as a cell adhesion molecule and provides specific binding with P-selectin glycoprotein ligand-1 located on WBCs' membrane. The channel was designed in serpentine manner as shown in Figure 2.4a. The microchannel was bonded on gold parallel strip patterns aligned at 15 degrees to the direction of the fluid flow and P-selectin was immobilized onto gold region as shown in Figure 2.4d. When the whole blood flowed through the channel, the target cells suspended in whole blood loosely bound with the P-selectin region and displaced along the strip and into the buffer streamline. At the end of the P-selectin strip, target cells migrated into the non-patterned region and were flushed away to the outlet. As a result, the neutrophils with 92% purity were retrieved from the outlet.

Although this technique yielded high efficiency, it requires additional substances such as P-selectin. The additional process for protein functionalization on gold electrodes is needed for device operation. Also, the use of gold patterning substrate can increase the cost of the device.

2.1.1.6 Inertial microfluidics

Recently, there is an emerging class of microfluidics for particle/cell separation termed inertial microfluidics. This technique exploits a lateral migration phenomena of particles at intermediate range flow (Reynold number (Re) between ~ 1 to ~ 100) (Amini et al. 2014; Di Carlo 2009). To understand the fundamental of this technique, inertial particle focusing in a straight channel is requisite and it can trace back to 1960, where Segre and Silberberg experimentally observed that randomly distributed particles flowing inside a straight tube became focused at ~ 0.6 times the channel radius (Segré and Silberberg 1961). This phenomena was corresponding to two dominant forces presenting in fluidic channel; 1) the wall-induced lift force which directs the particle away from the wall and 2) the shear gradient induced lift force which directs the particle away from the channel center (Amini et al. 2014; Di Carlo 2009) (Figure 2.5A). By exploiting inertial migration phenomena, researchers managed to design microfluidic devices for particle/cell separation applications. As shown in Figure 2.5B, Mach and Di Carlo designed inertial microfluidic device for bacteria filtration from blood (Mach and Carlo 2010). To facilitate bacteria removal, they used channel with aspect ratio of 3 (height/width). By using a high aspect ratio channel, the blood cells were highly focused close to channel walls enabling RBC collection from side outlets whereas unfocused

Figure 2.4 Affinity flow fractionation: (a) The two inlets and two outlets device for affinity flow fractionation designed by Bose et al. (2013) (b,c) Schematic of device at inlet and outlet sections. (d) Parallel gold stripes array coated with P-selectin (e) The neutrophil separation using this technique (Bose et al. 2013).

bacteria were eliminated via center outlet. They achieved 4 times RBC enrichment and 80% bacteria removal. Apart from inertial focusing in a high aspect ratio straight channel, contraction and expansion channels were also used by several literatures (Che et al. 2017; Dhar et al. 2018; Hur et al. 2011; Mach et al. 2011; Park and Jung 2009; Sollier et al. 2014). In this contraction and expansion design, the particles are inertially focused in a contraction region (high aspect ratio). As the particles entering expansion region, they experience only shear gradient induced lift force in the direction toward the vortex center (inside expanded region) (Figure 2.5C). For large size particles, they also experience larger lateral lift forces which induce lateral migration toward the expanded region where they continue recirculating (Park and Jung 2009). The cells trapped inside the vortex can then be collected by lowering flow rate. For instance, Sollier et al. (2014) utilized this technique for isolation of various cancer cell lines from diluted whole blood and achieved high cell viability of >80% and a capture purity of >75%, independent of cell line. However, the capture efficiency was only around 20% as the vortices can hold limited number of cells.

In addition to inertial lift forces, the variation in channel geometry can induce a secondary flow inside microchannel that can be exploited for particle manipulation. The notable examples are asymmetric serpentine channel (2007) and spiral channel (Bhagat et al. 2008b) which utilized curving structures to enable particle focusing or separation. As fluid traversing through curving channel, there is a Dean flow due to non-uniform velocity/inertia of fluid at different cross-sectional positions of the channel. Fluid at the channel center has higher velocity directed outwards compared to other positions (near channel walls). To conserve the mass, fluid at the channel center will recirculate outwards to near channel wall and the fluid at the channel wall recirculate inwards to the center, thereby generating two counter rotating vortices called Dean vortices (Amini

et al. 2014). This technique has been widely adopted in various blood related applications including CTC isolation (Hou et al. 2013) and blood cell separation (Hou et al. 2016; Ramachandraiah et al. 2017). Recently, our group reported a two inlets and four outlets spiral inertial microfluidics for neutrophil isolation with 80% purity from lysed blood (Hou et al. 2016) (Figure 2.5E). Additional sheath inlet provided two benefits; 1) the device featured buffer exchanger whereby sorted cells were resuspended into new medium. 2) the sample were pinched to the outer walls providing initialized cell position. Furthermore, the developed device also enabled separation of other leukocyte subsets; 1) Lymphocytes can be collected from the 3rd outlet. 2) Monocytes

Figure 2.5 Inertial microfluidics in particle separation (A) Inertial focusing effect in a straight channel (B) High aspect ratio straight channel for bacteria filtration by Mach *et al.* (Mach and Carlo 2010) (C) Microvortices in contraction and expansion channel (top) and microscopic images of particle trapping in expansion section (bottom) taken from Mach *et al.* (Mach *et al.* 2011). Arrow head indicates strength of shear-gradient lift force. (D) Spiral inertial microfluidics (E) Our previously developed device for leukocyte separation (Hou *et al.* 2016).

and neutrophils can be purified from PBMCs and lysed blood respectively and be collected from 2nd outlet.

Unlike straight channel designs, a major limitation for spiral or curvilinear devices is the difficulty for massive planar parallelization to achieve higher throughput. To facilitate high volume processing, the spiral stacking strategy has been reported by several groups (Khoo et al. 2014; Kwon et al. 2017; Miller et al. 2016a; Rafeie et al. 2016; Warkiani et al. 2014; Warkiani et al. 2015). Khoo *et al.* developed a multiplexed spiral microfluidic device for label-free enrichment of CTCs at ultra-high throughput (Khoo et al. 2014). The stacked device was fabricated by stacking three spiral channels vertically with shared common inlets and outlets (Figure 2.6A). To demonstrate the feasibility of this device, the authors processed blood samples (7.5 mL) from 10 healthy donors and 58 patients with metastatic breast or non-small cell lung cancer. The throughput was significantly enhanced (20-fold higher) which translated to processing 7.5 mL in less than 5 min with 100% detection sensitivity and high selectivity. Based on similar concept, Warkiani *et al.* utilized a stack of 40 trapezoidal spiral devices for Chinese hamster ovary cell (CHO cell) and yeast cells retention for bioreactor (Warkiani et al. 2015). Their system can operate at 240 mL/min. Rafeie *et al.* later demonstrated multiplexing of 16 trapezoidal spiral devices on one layer for blood plasma separation from diluted blood (Rafeie et al. 2016). They managed to achieve ~100% cell rejection ratio for ~0.5%-1% hematocrit at an optimal flow rate of 1.5 mL/min for a single device (24 mL/min for 16 devices). For stacking multiple devices, it should be noted that the top surface of the PDMS devices needs to be flat to prevent leakages between 2 consecutive layers. Precise alignment of the inlet and outlet ports is necessary to ensure that each device layer can receive equal flow distribution. In addition, stacking of multiple devices can suffer from slight pressure variance and might possibly affect

individual device performance. To overcome these problems, several groups have introduced custom design manifold to distribute fluid flow equally. For example, Miller *et al.* developed a manifold to control pressure-driven flow in each spiral device (Miller *et al.* 2016a). As shown in Figure 2.6B, they successfully incorporated 20 devices which can provide a large separation size range (2 – 300 μm) at a throughput of 1000 mL/min. Additionally, the system includes several unique sample processing features such as cascaded channels to enhance separation efficiency, and sample recirculation to increase purification and yield (Figure 2.6C).

Multiple stage spiral devices have been proposed to enhance sorting performance (Miller *et al.* 2016a; Robinson *et al.* 2017). Robinson *et al.* developed a two-stage spiral device for RBC depletion from diluted whole blood (2% Hematocrit (hct)). As a single stage spiral separation could not achieve high WBCs purity, they added another stage of spiral device at the end of first stage which has a bifurcation that equally splits into two smaller daughter spiral devices; one for subsequent RBCs exclusion and another one for transporting sorted RBCs to waste and balancing the fluidic resistances. They showed the device can effectively eliminate RBCs from diluted whole blood with 30-fold increase in RBCs depletion as compared to a single-stage spiral device (Robinson *et al.* 2017).

Besides multiple stage sorting, recirculation strategy is another attractive alternative for sorting of complex fluid such as blood or high cell concentration samples (Kwon *et al.* 2017; Miller *et al.* 2016a; Ryu *et al.* 2017). Briefly, sample recirculation is achieved by using peristaltic pump to feed output eluent (containing target cells) back to the inlet sample reservoir so that the suspension can pass through the device again. The recirculation strategy provides several benefits. First, it continuously dilutes and removes the waste materials that can otherwise affect the separation performance.

Secondly, it will enrich target cells into smaller volumes for subsequent processing without manual handling. In most cell sorting applications, the target cells in the samples are usually rare and do not contribute much to the sample concentration. Hence, the target cells recirculation will not compromise the separation performance. Figure 2.6D shows a recirculation strategy developed by Ryu et al. (2017). In this work, they utilized spiral inertial microfluidics with recirculation strategy to extract polymorphonuclear leukocytes from patient-derived airway secretion or sputum. As sputum is highly complex and heterogeneous among patient sample, a single step sorting is often insufficient to achieve satisfactory separation performance. Recirculation can purify target cell suspending medium while continuously eliminates mucin aggregates from sample. The reported device retrieved ~95% PMNs from sputum from 6 patients and provided superior performance over traditional Sputalysin (DTT) protocol (Figure 2.6A (*right*)). Kwon *et al.* recently described a closed-loop multiplexing spiral system for perfusion culture of Immunoglobulin G1 (IgG1) CHO cells with high IgG1 recovery rate (>99%), cell viability (>97%) and long term stability (18 – 25 days) (Kwon et al. 2017).

Figure 2.6 Integrated multiplexing spiral devices: **(A)** Photo of a multiplexed spiral device by stacking 3 spiral devices together for high throughput CTCs isolation. Adapted with permission from (Khoo et al. 2014), copyright 2014, PLoS; **(B)** Image of a stacked spiral system for large range particle separation. Reproduced with permission from (Miller et al. 2016a), copyright 2016, AIP Publishing; **(C)** Schematic illustration of cascaded sample processing using different spiral devices. Reproduced with permission from (Miller et al. 2016b), copyright 2016, Nature; **(D)** (left) Recirculation spiral microfluidics strategy. (right) Images and plots showing sputum and purified samples using their system (C-sep) and DTT protocol. Adapted with permission from (Ryu et al. 2017), copyright 2017, ACS Publications;

2.1.2 Active separation techniques

2.1.2.1 Magnetic-Based Separation

Like Magnetic Activating Cell-sorting (MACs), the same principle has been applied in microfluidics for separating suspended particles in liquid. The example use of this technique was reported by Deng et al. (2002). In this work, they fabricated an array of nickel posts into the PDMS substrate. The posts were used to trap particles based on their magnetic properties. However, this work did not use for separating blood components. Inglis et al. (2004) developed microfluidic device for the separation of WBCs from human blood using embedded patterns of ferromagnetic strip to provide the magnetic field in a microchannel. This device was capable of separating labeled WBCs from non-labeled RBCs.

For RBCs separation, non-labeling is also possible for magnetic-based separation as shown in many researches (Han and Bruno Frazier 2004; Han and Frazier 2006; Iliescu et al. 2008; Inglis et al. 2006). RBCs can be distinguished from other blood cells by its intrinsic magnetic property because of its hemoglobin containing Fe^{2+} . When RBCs are in deoxygenated form, each iron atoms in hemoglobin has 4 unpaired electrons rendering deoxygenated RBCs with paramagnetic property, while WBCs and other components have diamagnetic property. For example, Han and Frazier (2006)

Figure 2.7 Magnetic-based separation (A) Schematic of Han and Frazier (2006) device for magnetic separation: Single cascade paramagnetic separation device Under applied magnetic field, particles with different magnetic properties will align at different lateral positions enabling particle collection. (B) three cascade paramagnetic separation device was introduced to further enhance separation efficiency. Ferromagnetic wires are displayed in gray color.

reported a microfluidic device with ferromagnetic nickel wire integrated in the microchannel for RBCs separation. This work reported two designs which were single stage and three stage cascades shown in Figure 2.7A left and right respectively. When external permanent magnet was used to activate an internal nickel wire, RBCs were separated from whole blood sample to central outlet while WBCs were separated from whole blood sample to side outlets. For the single stage device, the result showed that the device can separate 91.1% of RBCs. While the three staged cascade device yielded better RBCs separation efficiency which is 93.5% and 97.4% of WBCs. Iliescu et al. (2008) developed a microfluidic utilizing this technique. Under the presence of magnetic field from the ferromagnetic layer, the RBCs were trapped while the WBCs and others were flushed out. This device was capable of trapping 95% of RBCs.

This technique provides efficient separation due to the specificity and selectivity of immunological reaction of labelled magnetic beads while the main disadvantage is the use of antibody labelling which requires additional sample preparation and expensive reagents.

2.1.2.2 Acoustrophoresis-based separation

Acoustic standing waves with high frequency excitation can be used to separate particles with different acoustic impedance based on their size, density and compressibility. Under an applied acoustic field, the suspended particle in fluid will be affected by the acoustic radiation force. If the diameter of the particle is smaller than the half wave length of the standing wave, the force will induce the particle to move toward either pressure node or pressure anti-node. In the microdevice, the acoustic force is generated by piezoelectric transducers by placing them on the outer wall of microchannel. When microchannel is excited at its fundamental frequency, the pressure node will be presented along the center while the pressure anti-node will be presented

alongside of the wall. The densities and compressibility of the particles and the medium play an important role in determining the direction the particle will move towards the pressure node or the pressure anti-node. There are several approaches that implemented this principle for blood cell separations.

Petersson et al. (2004) developed the microfluidic chip with three outlets to separate lipids from RBCs for intraoperative blood washing. As a result, this approach yielded separation efficiency greater than 95%. Later, this group furthered this method which was not only capable of removing lipids from RBCs but also featured buffer exchanger in which RBCs were transferred from contaminant medium to cleaner medium (Petersson et al. 2005). The result showed that 95% of RBCs with clean plasma can be retrieved from the outlet while 98% of contaminants were removed. Petersson et al. (2007) found that certain particles that cannot be separated in their original medium (plasma) could be separated by adding another different medium or modified original medium by density manipulation additive such as CsCl. Moreover, this work also demonstrated fractionation of multiple blood cell components which are RBCs, platelets and WBCs. The device is shown in Figure 2.8A. 86% of RBCs were collected at second and third outlets, while 92% of WBCs were collected at the third and fourth outlets and 79% of the platelets were collected at the fourth outlet. Even the results were not good because more than one type of components were detected at each outlet, there is a possibility that adding density manipulation additive and acoustophoresis could lead to separation of multiple cell types.

Lenshof et al. (2009) described Plasmapheresis chip for Prostate Specific Antigen detection. In this work, they found that in the short microchannel, much acoustic force is required to manipulate particles into the desired pressure nodes. High concentrations of sample also need more exposure time to the acoustic force to focus

the particle to the pressure nodes. Increasing the length of the microchannel and decreasing the flow rate can increase the separation efficiency of the device.

Aforementioned works are called bulk acoustic wave (BAW) - based acoustophoresis technique. A material choice for the microchannel of these devices has to have excellent acoustic properties. However, in microfluidic applications, commonly used materials such as PDMS do not have this property (Xia and Whitesides 1998). Another problem is that the transducer is bulky, hindering device integration and miniaturization (Shi et al. 2009). In order to address these problems, the standing surface acoustic waves (SSAW) based device was introduced by Shi et al. (2009). In this new design, the bulky transducer was replaced by surface interdigitated electrodes on a piezoelectric substrate such as LiNbO_3 . The author mentioned that the power consumption of new type transducer is less than that of the bulky transducers. Nam et al. (2011) implemented the standing surface acoustic waves for separation of platelets from whole blood sample. The device and whole processes are shown in Figure 2.8B. The device was capable of removing 99.9% of unwanted cells and 74.1% of platelets were collected from central outlet. The purity of collected platelets was 98%.

From the aforementioned ultrasonic separation approaches, many advantages of this technique are presented. First, the key advantage is no physical contact between the ultrasonic transducers and the suspension since physical contact between the transducers and suspension can alter the suspended particles. Second, this technique did not cause hemolysis or damage to the cells. On the contrary, this technique may not be suitable to distinguish two particles that have slightly size difference. It should be noted that high excitation power is typically required to enable acoustic separation inside the microchannel. As a consequence, joules heating and bubble generator inside microchannel can deteriorate performance of a device or even affect cell viability. To

mitigate this issue, a peltier element is commonly used to prevent a substrate from heating.

2.1.2.3 Dielectrophoresis separation

Inhomogeneous electric field will induce a dipole in a neutral particle suspended in the medium. Due to the presence of an inhomogeneous electric field, the net force will act on a suspended particle, and direct it to its equilibrium position where the dielectrophoresis (DEP) force is equal to another force presenting in microchannel such as drag force, gravitational force. The direction of the particle depends on whether the particle or the medium that is more conductive. If the particle is more conductive, it will move towards the high intensity electric field, this phenomenon is called Positive-Dielectrophoresis (pDEP). If the medium is more conductive, the particle will move away from the high intensity electric field, this phenomenon is called Negative-Dielectrophoresis (nDEP) (Figure 2.9A).

Figure 2.8 Acoustophoresis-based separation (A) Schematic of Petersson et al. (2007) device. On the top picture shows when different sized particles experience acoustic field generated by an ultrasonic transducer and align into different flow streams. On the bottom picture shows the outlet configuration of the device for collection of different sized particles. (B) SSAW based microdevice by Nam et al. (2011). Focused blood stream experiences surface acoustic wave generated by interdigitated transducer on LiNbO₃, different blood components align to the pressure node or anti-pressure node enabling platelet extraction from blood.

Gascoyne et al. (2002) reported two microfluidic designs to separate malaria-infected cells from blood. Malaria-infected RBCs are RBCs that host to malaria parasite exhibiting altered electrical property. With this subtle change in electrical property, malaria-infected RBCs can be separated from RBCs and other components in blood. They achieved 99.5% of normal RBCs trapping by pDEP whereas 90% the malaria-infected cells were continuously washed away.

Pommer et al. (2008) reported DEP based microfluidic device for platelets separation. By exploiting the fact that platelets are the smallest cells in blood, platelets were less affected by dielectrophoretic force compared to larger cells. The larger cells experienced a dielectrophoretic force that was sufficient for deflecting them into buffer stream which led them to waste outlet. This device can be used to separate around ~95% platelets from diluted blood at a throughput of $\sim 2.2 \times 10^4$ cells/second/channel. In Figure 2.9B, Piacentini et al. also demonstrated platelet separation using lateral liquid electrodes for inflicting nDEP and achieved >98% of platelet recovery and >98% of platelet purity (Piacentini et al. 2011). Nakashima et al. (2010) designed a microfluidic device for blood cell separation from plasma. When the AC was applied, the blood cells were trapped to the electrodes located on the microchannel by the nDEP force whereas the plasma flowed to plasma reservoir by the capillary force. As a result, 97% of blood cells were removed and 300 nL of plasma was retrieved from 5 μ L. Jones et al. (2011) utilized this technique with a sawtooth designed microchannel for RBCs separation. Platinum electrodes connected to a DC power supply were inserted into inlet and outlet. By activating DC potential, the RBCs were captured around the sawtooth edges.

Like any other active separation technique, the main disadvantage is the need of external power source. Nevertheless, it is still the promising technique because it offers versatility that allows one to move, separate or manipulate the target cells by simply

controlling the excitation pattern in addition to flow rate tuning. Another concern that has to be taken into account is the difficulty to separate cells whose crossover frequencies are too close. It is worth mentioning that nDEP manipulation requires cells to be in a low conductivity medium compromising cell viability (Pethig 2017).

2.1.2.4 Identification before active separation

This class of cell separation incorporates a detector (optical or image-based) to determine cell characteristics prior actuation to collect cells of interest. As FACs have been widely utilizing in life sciences, they are of great interest for miniaturizing the whole setup to smaller, cheaper device while still maintains equivalent performance. The first micro-fluorescence-activated cell sorting (μ FACs) was first proposed by Fu et al. (1999). Later Wolff et al. (2003) developed a high-throughput μ FACs integrated with various modules similar functionality to the macroscale counterpart. These modules included hydrodynamic focusing modules, the optical detection module and the pressure-driven sorting module (Figure 2.10). This device was used to separate fluorescent latex beads from chicken RBCs. The result showed that sample throughput of 12,000 cells per second with 100-fold enrichment can achieved from this device.

Figure 2.9 Dielectrophoresis-based microfluidics (A) Illustration of DEP working principle (Watarai 2013) (B) DEP device for platelet separation by liquid electrodes. Hydrodynamically focused mixed samples flows to DEP electrodes region where different particles experience different DEP forces enabling separation of platelet from RBC and WBC. (Piacentini et al. 2011)

Not only labelled cells can be distinguished by this technique but also the interested cell that has auto-fluorescence property can be distinguished as well as shown in the research of Emmelkamp et al. (2004). This work showed that granulocytes and RBCs can be separated from each other because of the different internal structure of granulocytes and RBCs.

Figure 2.10 Micro-fluorescence-activated cell sorting (μ FACs) from Wolff et al. (2003) (A) Device schematic (B) Scanning Electron Microscope (SEM) image of μ FACs showing 1. Focusing of sample stream (b) by sheath stream (a) 2. Optical interrogation window (c) 3. Reservoir for sorted cells (d).

Optical-based separation is an efficient technique to use because of its specificity and selectivity. However, utilization of this technique may have some difficulties. As this device is an integrated system of various modules, miniaturization of proper optical system or driven-system can be challenging. With the need of labelling, human handling is required for sample pre-treatment which the sample may be prone to contaminants. Labelling process may cause an irreversible effect to the target cell and label element may not be removed easily and may disrupt subsequent analysis. Moreover, the associated cost also increases due to the use of specific agents.

Table 2.1 provides comparison between different microfluidic cell separation techniques.

Table 2.1 Comparisons between different microfluidic cell separation techniques

	Technique	Features	Disadvantages	Ref
Passive separation	Microstructure	Label-free size-based separation <ul style="list-style-type: none"> Separation efficiency: 70-95% for WBCs Purity: 50-93.5% for WBCs 	<ul style="list-style-type: none"> Prone to clogging Precision fabrication Low throughput (5 μL/min) 	Ji et al. (2008)
	Biomimetic separation	Label-free size/deformability-based separation <ul style="list-style-type: none"> Capable of whole blood processing Separation efficiency: 80% for WBCs 	<ul style="list-style-type: none"> Low flow rate (0.5-5 μL/min) 	Tay et al. (2018)
	PFF	Label-free size-based separation <ul style="list-style-type: none"> Separation efficiency 80% for RBCs 	<ul style="list-style-type: none"> Prone to clogging Two pumps required for operation 	Takagi et al. (2005)
	DLD	Label-free deformability/size-based separation <ul style="list-style-type: none"> Separation efficiency: 91% for WBCs 	<ul style="list-style-type: none"> Precision fabrication Low throughput (2 μL/min) 	Zheng et al. (2005)
	AFF	Affinity-based separation <ul style="list-style-type: none"> Separation efficiency: 91% for WBCs 	<ul style="list-style-type: none"> Substrate with electrodes Non label-free with laborious sample preparation 	Bose et al. (2013)
	DFE	Continuous, label-free size-based separation <ul style="list-style-type: none"> High throughput: >1mL/min Separation efficiency: 80% for neutrophils Purity 90% for neutrophils 	<ul style="list-style-type: none"> Precision fabrication (sensitive to height) 	Hou et al. (2016)
Active separation	MACs	Affinity-based separation <ul style="list-style-type: none"> High specificity Separation efficiency: 80% for WBCs Throughput: 20 μL/h 	<ul style="list-style-type: none"> Non label-free with laborious sample preparation 	Inglis et al. (2004)
	Ultrasonic	Label-free compressibility/size-based separation <ul style="list-style-type: none"> Separation efficiency: 92% for WBCs Purity: 92% for neutrophils Throughput: 40 μL/min 	<ul style="list-style-type: none"> Substrate with electrodes/ BAW generator Joules heating 	Petersson et al. (2007)
	DEP	Label-free size/dielectric properties-based separation <ul style="list-style-type: none"> Separation efficiency: 99.5% for RBCs 	<ul style="list-style-type: none"> Substrate with electrodes Low viability when using non-conductive medium Joules heating 	Gascoyne et al. (2002)
	FACs	Label-free optical/fluorescence-based separation	<ul style="list-style-type: none"> Optical setup/ detector Non label-free with laborious sample preparation 	Emmelkamp et al. (2004)

2.2 Microfluidic impedance cytometry for single cell analysis

Not only microfluidics have been developed for cell separation, but they also have been utilized for various detection schemes either as bulk analysis or single cell analysis. Single cell analysis has gained considerable attention for biological assays and system biology in the past decade due to the increasing importance of studying cell populations that are highly heterogeneous, as well as sampling of complex biofluids

such as blood. Bulk measurement can only reflect the average value, leading to a loss of valuable information about rare sub-population (diseased cells or abnormal cells) present in the sample (Altschuler and Wu ; Sims and Allbritton 2007). In bio-related studies, coulter counter and FACS are widely used as high throughput cell counting and classification method. For the coulter counter, it detects a change in direct current (DC) or low frequency alternative current (AC) impedance signal caused by particle or cell passing through the detection region which can provide information about particle size (Coulter 1956; Hoffman et al. 1981). FACS is a more powerful technique and requires fluorescent cell labelling to enable counting, characterization and sorting based on optical characteristics. However, several drawbacks including laborious sample preparation, and expensive equipment and reagents (antibodies) significantly limit its use for point-of-care (POC) testing.

Of all techniques, impedance spectroscopy is an attractive alternative for label-free cell characterization based on size and intrinsic electrical properties of cellular components such as cell membrane and cytoplasm (Chen et al. 2015; Cheung et al. 2010; Petchakup et al. 2017; Sun and Morgan 2010). It has been exploited in various applications ranging from cell type identification (Hassan et al. 2017; Simon et al. 2016; Spencer et al. 2014a) to activation profiling (Evander et al. 2013; Rollo et al. 2017). This section will be about the historical progress of impedance spectroscopy of blood and its components.

The earliest work on blood cells using impedance spectroscopy is from Höber (1910, 1912). In this work, he studied the conductivity of RBCs at low frequency and high frequency. Also, he was the first researcher to report that RBCs comprising of poorly conducting cell membrane and conducting electrolyte solution (cytoplasm). Later, Fricke published several papers regarding the theoretical studies and experimental

studies of conductivity of cell membrane and capacitance of cell membrane (Fricke 1924, 1925a, b, c; Fricke and Morse 1925). The remarkable results from those set of studies were the estimated values of capacitance and thickness of cell membrane were 8.1 mFm^{-2} and 3.3 nm , respectively. He also published the measurement of impedance of rabbit's WBCs (Fricke and Curtis 1935).

Schwan reported three major dielectric dispersions in impedance spectra of biological cells in suspension namely α -dispersion, β -dispersion and γ -dispersion (Schwan 1994; Schwan 1956, 1963). In kHz range, there is α -dispersion which could originate from displacement of counterions around the charged shell of the cell. This is hard to measure because the interference of electrode polarization which contribute high impedance below 1 MHz (Schwan 1968b). β -dispersion occurring in MHz range is from the interfacial polarization of cellular components such as cell membrane. The polarization of protein and other organic molecules also contribute to different part of β -dispersion (Gabriel et al. 1996). Not only information of cells and cellular components can be obtained from this range but also RBC aggregation (Asami and Hanai 1992) and blood coagulation (Hayashi et al. 2010). Lastly, γ -dispersion found in above 1 GHz is due to dipolar relaxation of water bound molecules in the cytoplasm and the external medium (Kirkwood 1939).

Besides studying of RBC's dielectric properties, the lymphocytes' dielectric properties were investigated by several groups (Bordi et al. 1993; 1990; 1986). For example, Surowiec et al. (1986) reported the cell membrane capacitance and found that it can be used to discriminate human normal T-lymphocytes and B-lymphocytes. Other groups studied the dielectric properties of lymphocyte in stimulated conditions such as after mitogenic stimulation (Hu et al. 1990). Normal and malignant WBCs were reported

to have significant difference in dielectric properties as shown in several studies (Ermolina et al. 2001; Polevaya et al. 1999).

Several groups studied the deterioration of preserved blood by means of dielectric spectroscopy (Bordi et al. 2001; Hayashi et al. 2007). They found that the change in the dielectric response of the preserved blood caused by the morphological transition of RBCs. The chemical stimulus such as glucose was found to alter dielectric properties of RBCs as demonstrated in several literatures (Caduff et al. 2004; Di Biasio and Cametti 2010; Hayashi et al. 2003; Livshits et al. 2006; Livshits et al. 2009).

Beside lymphocytes, Irimajiri et al. (1987a); (Irimajiri et al. 1987b) studied cultured rat basophil and the effect of osmotic perturbation on its dielectric response. As a result, they found that changes in surface morphology and organelles' constitution and can affect the dielectric response. Recently, there were several literatures utilized impedance spectroscopy to examine the neutrophil extracellular traps (NETs) formation in cultured neutrophils which are relevant to the state of wound inflammation (Anna et al. 2015; Schröter et al. 2013; Urban et al. 2015).

Abovementioned approaches were done by measuring the dielectric properties of the suspension containing cell population. The acquired dielectric properties were representative of the whole population. For the case of blood, it contained various kinds of blood cells. The measurement of blood can only provide information of RBCs as they are major component in blood. With the advancement of microfabrication technology and measurement instrument, the researchers were able to study the dielectric properties of single cells. Single cells analysis gains popularity recently for several reasons. Even the cells have apparently identical appearance, they might have different dielectric

behaviours. Moreover, the analysis of cell properties becomes simpler as the cell-cell interaction does not have to be considered (Asami 2002).

The next part will provide the information about various dielectric and impedance spectroscopy measurement which have been utilized for single cells electrical characterization.

2.2.1 Dielectrophoresis and electrorotation

When a non-uniform external electric field is applied on cell in suspension, it can cause a movement of the suspending cell. There are two ways to imply the dielectric properties of cell which are DEP and electrorotation.

DEP, as mentioned previously, is a technique that exploits the difference between dielectric properties of cell and its suspending medium. If the cell is more polarizable than the medium, it experiences positive dielectrophoretic force and moves toward the high intensity electric field. On the contrary, if the cell is less polarizable than the medium, it experiences negative dielectrophoretic force which repels it from the high intensity electric field. This technique has been mostly used for sorting blood cells as mentioned in Chapter 2 as well as studies of RBCs properties (Cruz and García-Diego 1998). For dielectric profiling applications, Pethig et al. (2002) investigated T-lymphocytes treated with PMA and ionomycin. By tracking cells movement inside the channel, they found distinct difference in dielectric response of two samples. In 2009, Vykoukal et al. (2009) studied dielectric properties of each WBC subpopulation by using dielectrophoretic crossover frequency method. As a result, they showed that each type of white blood cells possesses distinct membrane capacitance.

In electrorotation, the rotating electric field is used which causes the cell to rotate in clockwise direction or counter-clockwise depending on which one is more

polarizable. This technique has been used to study dielectric properties of various blood cells such as RBCs (Donath et al. 1990; Georgieva et al. 1998) and lymphocytes (Ziervogel et al. 1986). In order to determine cells separation parameters for electrokinetic based separation, the studies of electrorotation of leukocyte subpopulation was carried out by Yang et al. (1999). For the neutrophil, Griffith and Cooper (1998) used electrorotation to study the changes in dielectric properties of neutrophil upon activation by phorbol myristate acetate (PMA).

2.2.2 Impedance cytometry

Characterization of electrical impedance at different frequencies provides important information about biological cells and the suspending medium, making impedance cytometry attractive tool for single cell analysis. (Schwan 1994; Schwan 1956, 1963). In the kHz range, there is α -dispersion which originates from the displacement of counterions around the charged shell of the cell. This is hard to measure as it is hindered by the effects of electrode polarization which leads to high impedance below 1 MHz (Schwan 1968a). β -dispersion occurring in MHz range arises from the interfacial polarization of cellular components such as the cell membrane. The polarization of protein and other organic molecules also contribute to different parts of β -dispersion (Gabriel et al. 1996). In addition to cells and cellular components information, dynamic studies of RBC aggregation (Asami and Hanai 1992) and blood coagulation (Hayashi et al. 2010) can be also obtained from this range. γ -dispersion above 1GHz is due to dipolar relaxation of water bound molecules in the cytoplasm and the external medium (Kirkwood 1939).

Advances in microfluidics and BioMEMs are important in the development of impedance cytometers as it enables manipulation of small fluid volumes. It also allows highly-sensitive measurement with the close proximity of microfabricated electrodes to

single cells in microchannels (Whitesides 2006; Yager et al. 2006). Besides reducing reagent and sample volume consumption and expensive equipment, a key advantage over the traditional methods is that sample preparation and impedance detection modules can be readily integrated in a single device, commonly known as lab-on-a-chip (LOC) for POC testing.

In this part, recent developments of impedance based microfluidic cytometry for biomedical research will be summarized and a brief overview of the working principles and designs of impedance based microfluidic cytometry will be discussed. For more detailed information, other excellent reviews are Morgan *et al.* (Morgan and Spencer 2014; Sun and Morgan 2010). and Chen *et al.* (Chen et al. 2015). Next, the focus will be on the diagnostics and phenotyping capabilities of impedance cytometry in different biomedical applications and reported works according to cell types including blood cells (leukocytes and RBCs), cancer cells, microbes and stem cells.

Design principles and theory

Electrical impedance is defined as the ratio between excitation voltage and response current of cell in suspension,

$$Z^* = \frac{V^*}{I^*} \quad (2.1)$$

Where Z^* is electrical impedance (Ohm), V^* is excitation voltage (Volt) and I^* is current response (Amp) and superscript * denotes complex number.

Various approaches have been utilized to simulate or to interpret impedance results of particle in suspension such as Finite Element Method (FEM), Maxwell's mixture theory (MMT) and equivalent circuit model (ECM). A comparison of three abovementioned approaches has been reported in literatures (Holmes et al. 2009; Hywel et al. 2007; Sun et al. 2008; Sun et al. 2007a; Sun and Morgan 2010; Tao et al. 2007).

Maxwell's mixture theory proposed by Maxwell describes dielectric properties of a particle in suspension (Maxwell 1881). The effective complex permittivity of a mixture can be determined by three key parameters, which are the complex permittivity of the cell, complex permittivity of its suspending medium and volume fraction, which is the ratio of volume of the cell to the volume of the channel.

$$\varepsilon_{eff}^* = \varepsilon_{out}^* \frac{2(1 - \varphi) + (1 + 2\varphi) \frac{\varepsilon_{in}^*}{\varepsilon_{out}^*}}{(2 + \varphi) + (1 - \varphi) \frac{\varepsilon_{in}^*}{\varepsilon_{out}^*}} \quad (2.2)$$

Where $\varepsilon^* = \varepsilon - j\frac{\sigma}{\omega}$ denotes complex permittivity, $j^2 = -1$, ω is the angular frequency, and φ is the volume fraction which is the ratio of volume of cell to volume of medium inside the detection channel. The subscript “eff”, “in” and “out” represent effective, inner and outer, respectively.

The above formula can be used to calculate a complex permittivity of inhomogeneous dielectric material which can be exploited to model dielectric properties of mixture of single cell for impedance cytometry. To determine complex permittivity of cell accurately, several models have been proposed to describe cell or particle based on its internal complexity such as particle, single shelled model (cell consisting of cytoplasm and cell membrane) and double shelled model (cell consisting of cytoplasm, cell membrane and nucleus or vacuole) (Hanai et al. 1979; Irimajiri et al. 1979; Willis 1980).

The impedance of the mixture containing cell (Z_{mix}) can be calculated from the following equation.

$$Z_{mix} = \frac{1}{j\omega\varepsilon_{mix}^*lG} \quad (2.3)$$

Where G is a cell constant which is to correct the effect of non-uniform electric field and fringing field. l is width of the channel. The calculation of cell constant of different electrode configurations was shown in previous literatures (Hong et al. 2005; Linderholm and Renaud 2005; Sun et al. 2008; Sun et al. 2007a; Sun et al. 2007b).

Electrode designs

In this section, three common configurations used in impedance based microfluidic cytometry namely coplanar electrodes, parallel electrodes, and constriction channel are presented. Figure 2.11A-C shows microfluidics impedance cytometers using coplanar electrodes design (Figure 2.11A), parallel electrodes design (Figure 2.11B), and constriction channel design (Figure 2.11C). Each design is based on similar detection principle, with excitation electrode and sensing electrodes embedded inside microfluidic channel to establish electrical measurement. As a cell flows between a pair of electrodes (A and C), the electric field between these two electrodes is disrupted, resulting in a current change that can be measured at point A. The current measured at this position corresponds to the impedance of cell and its suspending medium. To determine impedance of medium, the current at point B is also acquired simultaneously and the impedance of cell can be acquired from the difference between current at point A and at point C (Figure 2.11A). Typically, the setup consists of pre-amplifier, lock-in amplifier and data acquisition system (Figure 2.11D). The excitation signal is supplied to excitation electrode by function generator or lock-in amplifier, and sensing electrodes are connected to bridge circuit or trans-impedance amplifiers to measure current response of system. The amplifiers' output is connected to lock-in amplifier to demodulate current signal at excitation frequency. The data is sent to data acquisition system for post processing.

Figure 2.11 (A) (Left) Illustration of coplanar electrodes design and impedance signal response when a cell flows through the detection region. (Right) Impedance response at different frequencies carries different information regard the cell. Reproduced with permission from (Gawad et al. 2001), copyright 2001, Royal Society of Chemistry; (B) Illustration of parallel electrodes design. Reproduced with permission from (Cheung et al. 2005), copyright 2005, John Wiley and Sons; (C) Illustration of constriction channel design. Reproduced with permission from (Chen et al. 2011), copyright 2011, Royal Society of Chemistry; (D) Diagram shows the measurement setup. Reproduced with permission from (Sun et al. 2009), copyright 2008, Springer;

1. Coplanar electrode design

Coplanar electrode configuration was first proposed by Gawad *et al.* (Gawad et al. 2001). In this design, coplanar metal electrodes were integrated in microchannel and non-homogeneous electric field was generated. The authors carried out the simulation of cell impedance from equivalent circuit model and their simulation result showed that different parts of impedance spectra contain different information of cell components as presented in Figure 2.11A. Furthermore, they showed that opacity or a ratio of high frequency impedance magnitude to low frequency impedance magnitude does not

depend on position of cell in the channel. Since then, opacity is widely used as characterization parameter in impedance cytometry.

The fabrication process of coplanar electrodes design starts with the patterning of electrode layer on glass substrate. The channel layer is then fabricated or bonded on glass substrate, creating a microfluidic device with integrated electrodes. The whole process can be easily fabricated since only a single alignment is needed to guide electrodes to the desirable position inside the channel.

Due to non-uniform electric field created by coplanar electrode configuration, the impedance measurement relied on the vertical position of cell in the detection region considerably. To reduce the effect of vertical position of cell on impedance, another coplanar electrode configuration called liquid electrodes was used (Demierre et al. 2007; Mernier et al. 2012; Valero et al. 2010). In this case, the electrodes were placed at bottom of lateral channels perpendicular to main channel as shown in Figure 2.12A. As a result, homogeneous electrical field over the channel height was generated, mitigating the height dependence. However, this design had several drawbacks. Firstly, the sensitivity is poorer than traditional coplanar electrode design due to the increase in detection volume as the distance between the electrode pair needs to be placed far enough in order to generate homogenous electrical field across main channel. Secondly, the effect of lateral position rises due to fringing effect at edges of electrodes. In this work, they used dielectrophoretic force generated by liquid electrodes to focus the cell at the center of channel. Shaker *et al.* used the combination of conventional and liquid coplanar electrodes configuration shown in Figure 2.12B (Shaker et al. 2014). Longitudinal measurement and transverse measurement provide different characteristics that can be exploited to detect a shape of particle.

Besides down-scaling channel dimension to achieve higher sensitivity of coplanar electrodes, several techniques have been demonstrated to focus the cell to channel center or control the vertical position of the cell in the channel using DEP (Cheung et al. 2005; Evander et al. 2013; Holmes et al. 2007; Mernier et al. 2012; Morgan et al. 2006; Shaker et al. 2014) and hydrodynamic focusing (Barat et al. 2012; 2011; Rodriguez-Trujillo et al. 2008; Rodriguez-Trujillo et al. 2007a; Rodriguez-Trujillo et al. 2007b; Scott et al. 2008; Watkins et al. 2009).

For hydrodynamic focusing, there are two approaches, namely the 1D hydrodynamic focusing (Barat et al. 2012; 2011; Rodriguez-Trujillo et al. 2007a; Rodriguez-Trujillo et al. 2007b) and 2D hydrodynamic focusing (Rodriguez-Trujillo et al. 2008; Scott et al. 2008; Watkins et al. 2009). For these devices, the channels are typically larger and low conductivity sheath fluids such as deionized water (Evander et al. 2013; Rodriguez-Trujillo et al. 2008; Rodriguez-Trujillo et al. 2007a) or oil (2011; Evander et al. 2013) are used to achieve particle focusing. A three-inlets device was designed in which two additional focalisation lateral inlets were used to provide focusing stream for pinching sample stream. Not only does it allow single particles to flow through detection region, but the detection volume between electrodes can also be adjustable to fit a wide range of particles or cells sizes. Moreover, the use of a large channel greatly reduces the chance of channel blockage. Besides utilization of 1D hydrodynamical focusing, 2D hydrodynamical focusing was adapted in several devices (Rodriguez-Trujillo et al. 2008; Scott et al. 2008; Watkins et al. 2009), aiming to control vertical position of cell leading to better sensitivity than 1D hydrodynamical focusing.

To align particle to the center of channel using DEP, several designs such as top and bottom taper shaped electrodes (Morgan et al. 2006), coplanar deflecting electrodes (Cheung et al. 2005) and liquid electrodes (Mernier et al. 2012; Shaker et al. 2014) have

been reported. Noteworthy, the utilization of DEP focusing only provides the control of particle position in the channel whereas hydrodynamical focusing can control both the particle position and detection volume.

Besides abovementioned particle focusing techniques, the effect of particle position on impedance can be also be corrected by multiple electrodes design and signal processing as demonstrated recently by De Ninno *et al.* (De Ninno et al. 2017). Additional electrodes affect the measured signal profile which conveys information on particle position as well. Hence, the measured characteristic signal can be exploited to correct the signal of off-center particle leading to accurate particle sizing. However, introduction of additional electrodes covers a larger region in the channel and a higher particle coincidence (2 or more particles measured simultaneously) can occur if particle concentration is too high.

2. Parallel electrode design

Parallel electrodes configuration was first developed by Gawad *et al.* (Gawad et al. 2004). In this configuration, electrodes were placed at top and bottom or at sidewall of microchannel. Similar to previous design, two pairs of electrodes were used to measure impedance of cell passing between electrodes and impedance of the medium

Figure 2.12 (A) Illustration of liquid electrode design. Reproduced with permission from (Valero et al. 2010), copyright 2010, Royal Society of Chemistry; (B) Illustration of combination approach of conventional coplanar electrode design and liquid electrode design. Reproduced with permission from (Shaker et al. 2014), copyright 2014, Royal Society of Chemistry;

as depicted in Figure 2.11B. With parallel electrodes, electric field distribution was less divergent, leading to better sensitivity as compared to coplanar electrode design. However, this design also suffers from the measured signal dependence on cell position inside detection volume (Spencer and Morgan 2011).

The fabrication process is more complex as compared to coplanar electrodes design. For top and bottom electrodes configuration, two alignment steps are needed for aligning channel to electrode pattern and aligning two chips with electrodes together. Precise alignment is needed to make the measurement reproducible. For the sidewall electrodes configuration, sidewall electrodes were fabricated by electroplating followed by SU-8 channel fabrication on top of the electrodes. This can be done with single alignment. However, there is always a vertical gap between the sidewall electrodes and microchannel, resulting in an inhomogeneity of the electric field (Frankowski et al. 2015). This can possibly lead to a slightly poorer performance as compared to top-bottom configuration.

Several studies utilized hydrodynamical focusing (Bernabini et al. 2011) and DEP to control particle position inside microchannel. Additionally, multi-electrodes design used to correct the signal of off-center particles was proposed by Spencer *et al.* (2016). In this work, they used five pairs of parallel electrodes. However, unlike multiple coplanar electrodes design approach, this requires four transimpedance amplifiers to get parameters for correction, resulting in a more complicated setup.

3. Constriction channel design

Lack of direct contact between electrodes and cell can introduce current leakage issues, in which current tends to pass through high conductivity fluid surrounding the cell. In order to solve this problem, the constriction channel design was introduced by

Figure 2.13 (A) Illustration of constriction channel design with dual frequencies measurement (B) Equivalent circuit model and current paths at low and high frequency when constriction channel with cell and without cell. Reproduced with permission from (Zhao et al. 2013a), copyright 2013, Elsevier;

Chen *et al.* (Chen et al. 2011). In this design, the detection region was designed to be smaller than cell (Channel: $6\text{ }\mu\text{m} \times 6\text{ }\mu\text{m}$) as shown in Figure 2.13A. The Ag/AgCl electrodes placed at inlet and outlet were used instead of thin film electrodes on substrate. When cell was aspirated into the channel, the electric field across two electrodes was altered leading to the change in impedance which can be implied as impedance of cell. Moreover, mechanical properties such as cell deformability can be measured when the cell squeezes through the smaller channel, enabling multi-parametric mechanical and electrical cell characterization. Based on equivalent circuit model shown in Figure 2.13B, multi-frequencies measurement (at 1kHz and 100kHz) are used to determine size-independent electrical properties such as specific membrane capacitance ($C_{\text{specific membrane}}$) and cytoplasm conductivity ($\sigma_{\text{cytoplasm}}$). The drawbacks of this design are that it is prone to clogging and has lower throughput as compared to other designs.

The fabrication process of constriction channel design is simple as only single alignment is needed in channel fabrication process. The comparison of each design is shown in Table 2.2.

Table 2.2 Comparisons between different impedance cytometers design

Design		Advantages	Disadvantages
Coplanar electrodes design	Coplanar electrodes	Simple fabrication High throughput	Vertical position dependence Low sensitivity
	Liquid electrodes		Lateral position dependence Low sensitivity ¹
Parallel electrodes design	Top-Bottom configuration	High sensitivity High throughput	Vertical position dependence Complex fabrication
	Sidewalls configuration		Lateral position dependence Complex fabrication
Constriction channel		Simple fabrication High sensitivity Size independent electrical parameters Mechanical property characterization	Prone to clogging Low throughput

1 Sensitivity comparison of each design was presented in several literatures (Gawad et al. 2001; Mernier et al. 2012).

Blood-related applications

In this section, blood-related applications of impedance cytometers based on cell types are highlighted (Table 2.3).

Table 2.3 List of developed impedance cytometry applications based on cell type. WBCs: white blood cells; RBCs: red blood cells; TRAP: thrombin receptor activating peptide; PDMS: polydimethylsiloxane.

Category	Author	Summary	Characterization parameters
WBCs	Watkins <i>et al.</i> (2009)	CD4 T-cells counting using impedance cytometer with 2D hydrodynamical focusing	Impedance at 50kHz
	Holmes <i>et al.</i> (2009)	Discrimination of leukocyte subpopulation	Impedance signal at 1.7MHz and 503kHz
	Holmes <i>et al.</i> (2010)	Discrimination of T-cells and CD4 T-cells conjugated with CD4 beads	Impedance signal at 10MHz and 503kHz
	Watkins <i>et al.</i> (2011)	Differential count of CD4 T-cells by reverse-flow technique with integrated cell capture chamber	Impedance signal at 1.1MHz
	van Berkel <i>et al.</i> (2011)	Differential counting of blood cells using impedance cytometer with off-chip sample treatment	Impedance signal at 1.7MHz and 500kHz
	Han <i>et al.</i> (2011)	Evaluation of RBC lysis chip for differential counting of WBCs	Impedance signal at 1.7MHz and 444kHz
	Watkins <i>et al.</i> (2013)	Integrated sample treatment and cell capture chamber for differential CD-4 and CD-8 T-cell counting	Impedance at 303kHz and 1.7MHz
	Spencer <i>et al.</i> (2014)	Integrated optical detection coupling with compound air	Impedance signal at 2MHz and 500kHz

		lens for differential counting of blood cells	Fluorescence signal
	Frankowski <i>et al.</i> (2015)	Evaluation of parallel electrode designs for leukocyte sub-population counting	Impedance signal at 4MHz and 500kHz and fluorescence signal
	Hassan <i>et al.</i> (2016)	Integrated sample treatment and cell capture chamber for differential CD-4 and CD-8 T-cell counting	Impedance at 303kHz and 1.7MHz
RBCs	Gawad <i>et al.</i> (2001)	Discrimination of beads, RBCs and ghost RBCs	Impedance signal at 15MHz and 1.72MHz
	Cheung <i>et al.</i> (2005)	Discrimination of normal RBCs, ghost RBCs and glutaraldehyde fixed RBCs	Impedance signal at 10MHz and 602kHz
	Sun <i>et al.</i> (2007)	Utilization of maximum length sequences (MLS) for characterization of RBCs	Impedance spectrum up to 500kHz
	Kuttel <i>et al.</i> (2007)	Discrimination of RBCs, B. Bovis infected RBCs and ghost RBCs	Impedance signal at 8.7MHz
	Zheng <i>et al.</i> (2012)	Characterization of adult RBCs and neonatal RBCs	Transit time, amplitude ratio and phase shift at 100kHz
	Du <i>et al.</i> (2013)	Discrimination of different states of Plasmodium falciparum infected RBCs	Combination of phase shift and magnitude shift in impedance at 2MHz
	Evander <i>et al.</i> (2013)	Discrimination of RBCs, platelets and TRAP treated platelets.	Impedance signal at 284 kHz, 1.20 MHz, 2.39 MHz and 4.02 MHz
Tumors	Schade-Kampmann <i>et al.</i> (2008)	Discrimination of mouse fibroblast, adipocytes, human monocytes, dendritic cells and macrophages	Impedance signal at 2Mhz, 5Mhz and 14 MHz
	Nikolic-Jaric <i>et al.</i> (2009)	Discrimination of different sized polystyrene beads, yeast cells and chinese hamster ovary (CHO) cells	Capacitance at 1.5GHz
	Gou <i>et al.</i> (2011)	Discrimination of liver tumor cells at normal, apoptotic and necrotic status and leukemia cells	Resistance and capacitance change at 100kHz
	Chen <i>et al.</i> (2011)	Characterization of osteoblasts and osteocytes / EMT6 cells and EMT6/AR1.0 cells	Cell elongation, transit time and impedance amplitude ratio at 100kHz
	Mernier <i>et al.</i> (2012)	Utilization of lateral liquid electrodes for focusing and for discrimination of live and dead CHO cells	Impedance signal at 500kHz and 15MHz
	Zheng <i>et al.</i> (2012)	Characterization of 3249 AML-2 cells and 3398 HL-60 cells	Membrane capacitance and cytoplasm conductivity
	Zhao <i>et al.</i> (2013)	Characterization of kidney tumor cells (786-O) and vascular smooth muscle cells (T2)	Membrane capacitance and cytoplasm conductivity
	Zhao <i>et al.</i> (2013)	Characterization of lung cancer cell lines (CRL-5803 cells and CCL-185)	Membrane capacitance and cytoplasm conductivity
	Zhao <i>et al.</i> (2014)	Characterization of various kinds of tumor such as 95C and 95D / 549 and A549 CypA-KD	Membrane capacitance and cytoplasm conductivity

Kirkegaard <i>et al.</i> (2014)	Characterization of HeLa cells and Paclitaxel treated HeLa cells	Impedance signal at 1.57MHz and 82kHz
Spencer <i>et al.</i> (2014)	Detection of MCF7 cells spiked in whole blood	Impedance signal at 4MHz and 500kHz
Bürigel <i>et al.</i> (2015)	Inversion of flow direction enabling impedance measurement of HeLa and CHO-K1 cells before and after electroporation	Impedance spectra from 20kHz to 20Mhz with 8 steps
Zhao <i>et al.</i> (2015)	Characterization of mouse tumor cell lines (A549 and H1299)	Membrane capacitance and cytoplasm conductivity
Huang <i>et al.</i> (2015)	Characterization of normal PC-3 cells and PC-3 cells with membrane staining and/or fixation (4 conditions)	Membrane capacitance and cytoplasm conductivity
Yuan <i>et al.</i> (2016)	Utilization of AgPDMS as sidewall electrodes for discrimination of AML-2 and HL-60	Impedance signal ranging from 11kHz – 6MHz
Babahosseini <i>et al.</i> (2016)	Study the effect of different drug delivery approaches on electrical properties of MDA-MB-231	Impedance at 1kHz, 10kHz, 100kHz and 1MHz
Xie <i>et al.</i> (2017)	Discrimination of apoptotic, necrotic and live HeLa cells	Conductance and susceptance at 1MHz

White blood cells

Holmes *et al.* proposed impedance labeling technique for counting of CD4+ T-cells (Holmes and Morgan 2010). Anti-CD4 antibody coated beads (1.8 – 2.4 μm) were mixed with lysed whole blood and bound to the monocyte and CD4 expressing (CD4+) T-cells. As a result, the population of CD4+ cells were larger due to the beads bounded on their surfaces, resulting in an increase in opacity (10MHz and 0.5MHz) and impedance signal at 0.5MHz (Figure 2.14A). This method of using impedance labeling enables enumeration of sub-population. Spencer *et al.* also described a novel sheathless microfluidic cytometer with on-chip waveguide (Figure 2.14B (left)) that can measure four parameters; fluorescence signal, large angle side scatter and impedance at two different frequencies (0.5MHz and 2MHz) (Figure 2.14B (right)).

In another application for whole blood enumeration, van Berkel *et al.* developed an integrated microfluidic platform with sample pretreatment module for a 3-part

differential leukocyte counting together with RBCs and platelets counting (van Berkel et al. 2011). Figure 2.14C shows sample pretreatment design. Blood sample supplied to the sample pretreatment module was divided into two branches; 1. Dilution process of subsequent RBC and platelets counting based on impedance signal at 0.5MHz. 2. RBC

Figure 2.14 (A) Plot of opacity (10MHz/503kHz) versus impedance signal at 503kHz for white blood cells population after addition of CD4 beads. Reproduced with permission from (Holmes and Morgan 2010), copyright 2010, American Chemical Society; (B) (Left) Schematic of impedance cytometer with on-chip waveguide (Right) 3-D scatter plot of side scatter impedance and fluorescence for CD4 labelled white blood cells population. Color represents opacity magnitude. Reproduced with permission from (Spencer et al. 2014a), copyright 2014, Royal Society of Chemistry; (C) (a) Schematic of sample pretreatment module proposed by van Berkel *et al.* (van Berkel et al. 2011) for whole blood processing prior impedance detection. The design includes two pathways 1. Sample dilution followed by red blood cells and platelets counting 2. Red blood cells lysis and quenching followed by white blood cells differential counting. Reproduced with permission from (van Berkel et al. 2011), copyright 2011, Royal Society of Chemistry; (D) Schematic of on-chip sample pretreatment with capture chamber for differential counting of CD4 or CD8. 1. Inlets for loading of whole blood and reagent solutions (lysing buffer and quenching buffer). 2. Lysing followed by quenching 3. Entrance counting 4. CD4 or CD8 capture chamber 5. Exit counter. Reproduced with permission from (Hassan et al. 2016), copyright 2016, Nature Publishing Group;

lysis and quenching followed by WBCs discrimination based on opacity (1.7MHz and 0.5MHz) and impedance signal at 0.5MHz.

Recently, Hassan *et al.* reported a microfluidic impedance cytometer for simultaneous CD4+ and CD8+ T-cells counting (Hassan et al. 2015; Hassan et al. 2016; Watkins et al. 2013). In this design (Figure 2.14D), they included on-chip sample preparation and capture chamber specifically designed for capturing CD4+ or CD8+ T-cells. Cell population was electrically characterized before and after capture chamber, providing the number of cells captured in the capture chamber. The device was clinically validated in a cohort of healthy subjects and HIV+ patients, and they showed that the microfluidic measurements were strongly correlated to flow cytometry analysis.

Red blood cells and platelets

Gawad *et al.* demonstrated the discrimination of normal RBCs and ghost RBCs (their cytoplasm replaced by hypotonic solution) based on impedance signal at 15 MHz, indicating the differences was due to cytoplasm conductivity between both RBCs populations (Gawad et al. 2001).

Cheung *et al.* used parallel electrodes design to distinguish three kinds of RBCs, healthy, ghost and glutaraldehyde-fixed, at different concentrations (Cheung et al. 2005). In this study, they showed the identification of RBCs and glutaraldehyde-fixed RBCs based on impedance signal at 10MHz and 602kHz (Figure 2.15A). The impedance signal at 10MHz of fixed RBCs was higher than that of normal RBCs, indicating a decrease in cytoplasm conductivity or increase in opacity.

Parasite invasion of RBCs can alter dielectric properties of RBCs in malaria (Du et al. 2013; Gascoyne et al. 1997; Küttel et al. 2007). Kuttel *et al.* used coplanar electrodes design impedance cytometer to detect *Babesia bovis* infected RBCs (Küttel

et al. 2007). Figure 2.15B shows the difference in impedance signal at 8.7MHz of normal RBCs and *Babesia bovis* infected RBCs.

For platelet analysis, Evander *et al.* demonstrated the detection of RBCs and platelets as shown in Figure 2.15C (top). Moreover, the group also successfully classified non-activated platelets from thrombin receptor activating peptide (TRAP) activated platelets based on discriminant analysis from impedance signal at 4 frequencies (284 kHz, 1.20 MHz, 2.39 MHz and 4.02 MHz), which will be useful to study thrombosis or platelet dysfunctions (Figure 2.15C (bottom)).

Cancer cells and tumors

Figure 2.15 (A) Scatter plot of signal amplitude at 10MHz versus 602kHz for 5,6μm beads, red blood cells and glutaraldehyde fixed red blood cells at different concentrations. Reproduced with permission from (Cheung et al. 2005), copyright 2005, John Wiley and Sons; (B) Real part of signal amplitude versus imaginary part of signal magnitude of different kinds of red blood cells (ghost, normal, parasite infected). Reproduced with permission from (Küttel et al. 2007), copyright 2007, Elsevier; (C) (Top) Histogram shows the distribution of in-phase amplitude from platelets and red blood cells. (Bottom) Scatter plot of discriminant analysis of non-activated platelets and TRAP activated platelets. Reproduced with permission from (Evander et al. 2013), copyright 2013, Royal Society of Chemistry;

Figure 2.16 (A) Scatter plot of opacity (2MHz/500kHz) and diameter (impedance signal at 500kHz) for leukocytes and MCF7 cells. Color represents fluorescence signal. Reproduced with permission from (Spencer et al. 2014b), copyright 2014, AIP Publishing LLC; (B) Scatter plot of $C_{\text{specific membrane}}$ versus $\sigma_{\text{cytoplasm}}$ for A549 (mouse I) and H1299 (mouse IV). Reproduced with permission from (Huang et al. 2015), copyright 2016, Nature Publishing Group;

Several impedance cytometers were developed to characterize various types of tumor and cancers (Chen et al. 2011; Huang et al. 2015; Spencer et al. 2014b; Zhao et al. 2013a; Zhao et al. 2013b; Zhao et al. 2016; Zhao et al. 2014; Zheng et al. 2013).

Previous studies reported distinct differences in dielectric properties of WBCs and tumor cells, which generally have larger membrane capacitance and size (Becker et al. 1995; Gascoyne et al. 2013). Due to these differences, Spencer *et al.* demonstrated the use of parallel design microfluidic cytometer to distinguish breast tumor cells (MCF-7) from leukocytes when spiked in whole blood (Figure 2.16A) (Spencer et al. 2014b).

Zhao *et al.* characterized H1299 and A549 cells using a constriction channel design (Zhao et al. 2016). Specific membrane capacitance and cytoplasm conductivity of each population were acquired, which enable rapid discrimination of two tumor types ((Figure 2.16B).

2.3 Integrated microfluidic platform

Since the emergence of microfluidics, several microtechnologies for cell sorting and analysis have been proposed. For example, Toner et al. developed a microfluidic chip named CTC-iChip that integrated three different microfluidic sorting techniques (DLD, inertial focusing and magnetophoresis) to achieve isolation of circulating tumor cells without on-chip detection capabilities as shown in Figure 2.17A (Karabacak et al. 2014; Nagrath et al. 2007). Additionally, coupling spiral microfluidics with other cell sorting modalities was recently proposed by Nivedita *et al.* (Nivedita et al. 2017). In this work, they described a novel integrated platform which comprised of a spiral separator and a 2nd-stage cell sorter based on lateral cavity acoustic transducer (LCAT). Briefly, angled lateral channel array was designed to trap air as depicted in Figure 2.17B. Acoustic field was applied to the device which resulted in oscillation of the air/liquid interface and generation of microstreaming vortices. When larger particles approached these microvortices, they would be trapped into the circulating inner streamlines as smaller particles followed along the outer streamlines with the bulk flow (Nivedita et al. 2017). By adjusting excitation voltage of acoustic actuator, the device can selectively capture different sized particles to further enrich target cells. As proof-of-concept, they applied the device to trap larger monocytes (>18 μm) from 10 $\times$ diluted blood. Smaller cells (RBCs and smaller WBCs) and intermediate size cells (<18 μm) were eliminated to outlet 1 (spiral outlet) and outlet 2 (LCAT), while enriched larger monocytes remained trapped inside LCAT part. By flushing PBS through the channel, they were able to retrieve and concentrate target cells with an enrichment of 987-fold. They also demonstrated separation of DU-145 cells (>16 μm) from highly heterogeneous DU-145 population (7 – 28 μm) and achieved 91.7% purity and 67.5% recovery rate of target cells. Noteworthy, this seminal work showed the unification of two separation

techniques operating at different flow rates (1500 $\mu\text{L}/\text{min}$ and 25 $\mu\text{L}/\text{min}$). This was done by adjusting the pressure drop/hydraulic resistance of the spiral outlet and transition region between 2 devices. Another simple modification that can assist in enrichment of cells was proposed by Wang *et al.* (Wang et al. 2015) whereby they added a membrane filter at the outlet of the spiral device to further enrich CTCs for immunostaining.

For blood cell sorting applications, it remains challenging to use whole blood as a direct input for spiral devices due to the high RBCs concentration (~ 5 billion/mL) and secondary cell-cell interactions are known to have adverse effects on sorting performance (Hou et al. 2011; Ramachandraiah et al. 2017). This makes off-chip sample pre-processing (dilution or RBC lysis) a prerequisite prior spiral separation. Recently, Ramachandraiah *et al.* proposed an integrated spiral device for leukocyte fractionation with on-chip RBC lysis (Figure 2.17C) (Ramachandraiah et al. 2017). Whole blood and hypotonic solution (deionized water) were introduced into the device to selectively lyse the RBCs. The integrated lysis chamber with expansion chambers helped increase residence time for proper RBCs lysis. The lysed blood was then washed with sheath buffer stream (PBS) to prevent negative effects such as leukocytes swelling. Finally, the focused sample stream proceeded through the double spiral channel to fractionate out granulocytes, monocytes, and lymphocytes with a purity of 86%, 41% and 91% respectively. Such integrated device is favorable for point of care systems as it facilitates user operation and is less time-consuming process compared to sequential sample processing. Furthermore, it reduces cell loss that can occur during sample handling between each process. It should be noted that the abovementioned device required 3 syringe pumps to operate which can be a major drawback for point of care applications.

Although various integrated platforms have been demonstrated, they were designed to achieve higher sorting efficiency. Hence, there is a critical lack of “sample-in-answer-out” integrated platforms which is crucial for clinical applications. As mentioned earlier in impedance detection section, a notable example of integrated platform is from Bashir *et al* (Hassan et al. 2017; Hassan et al. 2016). The chip combines sample pre-processing (RBC lysis), capture chamber and impedance identification before and after capture chamber. This approach can enumerate specific leukocytes/cells by differential counting of cells entering and exiting capture chamber immobilized with antibodies. However, it requires antibody immobilization, and considerable device preparation efforts prior use. Moreover, the cells are not suitable for downstream activation analysis due to RBC lysis and RBC fragments co-flowing through the detection region with target cells can adversely affect the impedance detection. Lastly, it is worth mentioning that Lab-on-a-CD/disc is another well-established platform for “sample-in-answer-out” devices (Henderson et al. 2016). The platform offers great versatility since it can host various on-chip functionalities such as plasma separation, dilution and detection as demonstrated in Ducrée *et al* (Godino et al. 2014). However, this platform is primarily used for biomolecule detection and not cell-based analysis. Hence, they do not facilitate cell collection for downstream analysis.

Figure 2.17 Integrated microfluidic platform (A) Integrated platform for circulating tumor cells sorting by Toner *et al.* (Karabacak *et al.* 2014) (B) (left) Schematic of integrated spiral device with sequential lateral cavity acoustic transducer. (right) Microstreaming vortices in LCAT channel for particle trapping. Adapted with permission from (Nivedita *et al.* 2017), copyright 2017, Royal Society of Chemistry; (C) (top) Workflow and image of the integrated spiral device with on-chip RBC lysis. Reproduced with permission from (Ramachandraiah *et al.* 2017), copyright 2017, Royal Society of Chemistry;

2.4 Summary

This chapter provides a broad literature review on microfluidic cell separation, impedance cytometry and integrated platform. In term of microfluidic cell separation, various techniques with different key features have been proposed. Of all techniques,

inertial microfluidics stand out as continuous label-free size-based cell separation with high throughput ($>1\text{mL/min}$), high purity, minimal cell activation as well as buffer exchange capability. All these attributes make it favorable for leukocyte separation. For cell detection, single cell impedance cytometry is an emerging label-free technique based on electrical properties of a single cell. Several designs were proposed. Constriction channel design may have a lot of attractive features especially size-independent intrinsic electrical properties, but they are prone to clogging thereby limiting their use in a continuous manner. Parallel electrode configuration (top-bottom) may offer a good sensitivity but it restricts the device height to the detection channel height thus limiting on-chip integration with other modules that require a different height (such as DFF). Lastly, coplanar electrode configuration comes with compromised sensitivity, but they are simple to fabricate and can be easily combined with other microfluidic modules. Various applications have been demonstrated but only limited to cell identification. Hence, there is a research gap in leukocyte activation profiling which would be essential for immunology research and development of a novel diagnostic platform. In term of integrated platform, several exceptional platforms have been reported. However, they relied on antibodies labeling to tag or capture target cells for separation or identification. Besides being expensive, laborious and time-consuming sample labelling might not be suitable for the POC setting.

CHAPTER 3 MATERIALS AND METHODS

3.1 Device design principle

3.1.1 Combinatorial strategy for leukocyte phenotyping

To perform selective leukocyte phenotyping, leukocyte separation prior impedance detection was proposed.

For leukocyte separation, the previously developed DFF device was shown in Figure 3.1A (Hou et al. 2016). As mentioned in Chapter 2, this technique relies on the interplay between inertial focusing at intermediate range flow (Re between ~ 1 to ~ 100) and secondary dean flow inside curvilinear channel. For inertial focusing, a migration of particles in straight microchannel happens when $a_p/D_h \geq 0.07$ (where a_p is particle diameter and D_h is hydraulic diameter of microchannel defined as $2w \times h/(w + h)$). This is corresponding to two opposing forces presenting in microchannel; 1) the wall-induced lift force which directs the particle away from the wall and 2) the shear gradient induced lift force which directs the particle away from the channel center (Amini et al. 2014; Di Carlo 2009). As a result, a particle will migrate to equilibrium position (~ 0.6 times of D_h given a long enough microchannel) and the net inertial force is given by following expression (Asmolov 1999).

$$F_L = \rho G^2 C_L a_p^4 \quad (3.1)$$

Where ρ is the density of fluid medium, G is the shear rate of the fluid given (s⁻¹) by $G = U_{\max}/D_h$, $U_{\max}$ is the maximum fluid velocity and C_L is the non-dimensional lift coefficient which is a function of the particle position across the channel cross-section and Reynolds number (Re).

Besides inertial focusing, there exists a secondary flow inside curved microchannel. Due to non-uniform fluid velocity/inertia at different cross-sectional positions of the channel, it forms two symmetrical counter-rotating Dean vortices at the top and bottom half of the channel. This dean vortex imposes additional Dean drag force (F_D) on a particle traversing through curved channel as follows (Ookawara et al. 2006).

$$F_D = 5.4 \times 10^{-4} \pi \mu D e^{1.63} a_p \quad (3.2)$$

Where De is Dean number which given by $De = Re \sqrt{\frac{D_h}{2R}}$ and R is the radius of curvature.

The interplay between F_D and F_L renders differential lateral positions for different sized particles. The design criteria for successful separation of a target cell are as followed. 1) If a larger particle that complies $a_p/D_h \geq 0.07$, the particle equilibrium position depends on the ratio between net lift force and dean force ($\frac{F_L}{F_D}$); the largest particle will be the closest to the inner wall (Kuntaegowdanahalli et al. 2009). 2) On the contrary, for a smaller particle ($a_p/D_h < 0.07$), there will be no equilibrium position for the particle, and it will circulate in the Dean vortices. The calculations below can be used to determine lateral migration velocity (U_L), channel length (L_1) and migration length (L_D) to further obtain an optimal spiral length and flow rate that can provide distinct lateral positions enabling separation of smaller particles from larger particles (Bhagat et al. 2008a).

$$U_L = \frac{\rho U_{max}^2 a_p^3 C_L}{3\pi \mu D_h^2} \quad (3.3)$$

$$L_1 = \frac{U_f}{U_L} L_M \quad (3.4)$$

$$L_D = \frac{U_f}{U_{Dean}} L_1 \quad (3.5)$$

Where $U_{Dean} = 1.8 \times 10^{-4} De^{1.63}$ and U_f is average fluid velocity.

In this work, a spiral microchannel ($500 \mu\text{m}$ (w) $\times$ $115 \mu\text{m}$ (h)) has a radius of $0.9 - 1 \text{ cm}$ with a total length of 10 cm (Figure 3.1A). The sample ($75 \mu\text{m}$ wide) and sheath ($425 \mu\text{m}$ wide) are located at the outer and inner wall of the channel, respectively (Hou et al. 2016). The use of two inlet is to hydrodynamically confine sample stream to the outer wall. This also provides a control over particle initial position. By exploiting this technique, continuous multiplexed label-free separation and collection of different leukocyte subtypes with high purity ($\sim 80\%$) in a high throughput manner ($>1 \text{ mL/min}$) can be achieved. For this particular device, neutrophils or monocytes, lymphocytes can be collected from 2nd and 3rd outlets, respectively. Then the impedance profiling of sorted leukocyte subsets was performed on another platform. Of note, the device can

Figure 3.1 Device layout for combinatorial strategy for leukocyte phenotyping (A) device layout of DFF sorter (left) and schematic illustration of DFF separation (right) (Hou et al. 2016) (B) device layout of impedance cytometer (left) and schematic illustration of impedance detection.

also operate various common blood samples. Typically, RBC lysis was performed to lyse RBCs in blood to get neutrophil rich sample for neutrophil sorting while Ficoll density centrifugation was performed to get PBMCs (lymphocytes and monocytes) for monocyte and lymphocyte sorting.

For impedance detection, a two-inlets microfluidic channel was used. As shown in Figure 3.1B, one inlet is for sample solution, while another is for sheath buffer which is to hydrodynamically pinch a sample stream into single cell stream at the channel center. This also helps to prevent cells from sticking to the wall and provide a possibility of increasing the detection sensitivity by pinching cell stream with non-conductive fluids (as mentioned in Chapter 2). The detection channel located at the center of the device has cross-sectional dimension of 30 μm and 20 μm in width and height respectively. The three coplanar electrodes (20 μm in both width and gap) was used for differential measurement. Both electrode and channel dimensions are slightly larger than cells of interest (leukocytes 7-15 μm) to achieve high detection sensitivity (cell to detection channel dependent).

3.1.2 Integrated platform for leukocyte sorting and impedance profiling

Abovementioned strategy was realized into an integrated platform on a single chip as illustrated in Figure 3.2. The platform consists of three parts namely DFF sorter, flow rate reduction and impedance detection. The first part is DFF sorter which is similar to the above-described device. To account for flow rate mismatch between DFF sorter and impedance detection, we implemented two-staged asymmetric serpentine channel for flow rate reduction (FRR). Asymmetric serpentine channel proposed by (Di Carlo et al. 2007) were used to inertially focus randomly dispersed particles from a previous stage (DFF) will be focused and ordered close to the channel center while excess fluid can be removed from the side channels, thus effectively reducing flow rate before impedance detection. As particles ($ap/Dh > 0.07$) traverse through a small curved channel, they undergo lateral migration and continue to a big curved channel where they undergo

Figure 3.2 Integrated platform for leukocyte subset sorting and impedance phenotyping Layout of the integrate platform (top) Schematic illustrations depicting target cells flow behavior at different stages namely the particle sorting (DFF) (stage 1), flow rate reduction (FRR) (stage 2), and impedance detection (stage 3) (bottom).

lateral migration again but in an opposite direction. After traversing though several pairs of asymmetric structures, particles will align close to the channel center. The major benefit for the asymmetric serpentine channel is that inertial focusing can be achieved in a shorter length compared to the straight channel due to secondary dean flow (Di Carlo et al. 2007). The flow rate reduction ratios for 1st stage and 2nd stage are 3 and 2, respectively. To achieve desired flow rate reduction, a hydraulic circuit network of the device was determined as shown in Figure 3.3 and used for designing of device layout. Similar to Ohm's law for electrical circuit ($\Delta V = IR$), pressure drop between microchannel (ΔP) can relate to volumetric flow rate (Q) and hydraulic resistance (R_h).

$$\Delta P = QR_h \quad (3.6)$$

For the hydraulic resistance, it is given by

$$R_h \cong \frac{8\eta L}{r_h^2 A} \quad (3.7)$$

Where η is L is channel length. r_h is hydraulic radius of the channel which is defined as

$$r_h = \frac{2A}{P} \quad (3.8)$$

Where A and P are the cross-sectional area and the length of the perimeter of the microchannel respectively.

By application of conservation of mass at every fluidic node or branched channel, one can determine the flow rate distribution in the microfluidic network (similar to Kirchhoff's current law).

$$\sum Q_{in} - \sum Q_{out} = 0 \quad (3.9)$$

To simplify the analysis, we assume that the hydraulic circuit of our system is purely resistive (ignore the effect of the channel compliance). Figure 3.3 shows the

Figure 3.3 Hydraulic circuit of the integrated platform: Hydraulic resistances of the microfluidic network are designed such that the flow rate is greatly reduced by 40× prior impedance detection channel.

schematic of hydraulic analysis of the integrated platform using PSpice (Cadence) in which we use calculated hydraulic resistances to design meandered channels to control flow rate distribution to each stage.

After FFR stages, the target cells then proceed to a lower height impedance interrogation region (30 μm (w) $\times$ 20 μm (h)) where their impedance characteristics are measured.

3.2 Device fabrication

A single layer SU-8 mold was fabricated using standard photolithography process. Briefly, SU-8 2010 (Microchem) was spin-coated on a silicon wafer (Bonda Technology) and pre-baked at 65°C for 1 min and 95°C for 3.5 min. The coated wafer

was then exposed to UV using mask aligner (MA6; Karl Suss) and post-baking was performed at 65°C for 1 min and 95°C for 4.5 min before developing with SU-8 developer (Microchem). Before PDMS (Dow Corning) casting, the patterned wafer was silanized with trichloro(1H,1H,2H,2H-perfluorooctyl)silane (Sigma Aldrich, USA) overnight to ease PDMS releasing from the wafer. For the PDMS casting, a prepolymer and curing agent was mixed in a 10:1 ratio (w/w), vacuum degassed and poured over the fabricated SU-8 mold before curing at 80 °C for 2 h. The solidified PDMS slab was then gently peeled from the mold and a 1.5mm biopsy puncher (Harris Uni-Core™) was used to create the inlet and outlet holes. For the electrode fabrication, AZ9260 (Clariant) was used to define electrode pattern on a quartz wafer prior metal deposition by sputtering of 25 nm chromium and 100 nm gold. The channel was designed to be 30 μm (w) $\times$ 20 μm (h) for the electrical detection region. The PDMS and electrode substrate were irreversibly bonded by air plasma treatment using plasma cleaner (Harrick Plasma Cleaner) and heated on hotplate at 100°C for 1 h to ensure firm bonding.

For the integrated platform, a double layer SU-8 mold of 20 μm and 115 μm height was fabricated using standard photolithography process. The fabrication of the first layer was similar to abovementioned section. To define the second layer of 115 μm , SU-8 2100 (Microchem) was spin-coated on the same wafer at 2650 rpm and pre-baked at 65°C for 5 min and 95°C for 22 min. The mask features were aligned onto the coated wafer prior to UV exposure using mask aligner and post-baking was performed at 65°C for 5 min and 95°C for 10 min before developing with SU-8 developer. Then the wafer was hard baked at 120°C for 30 min and left to cooldown to room temperature.

3.3 Leukocyte preparation

3.3.1 Leukocyte isolation

Depending on the leukocyte of interest, the whole blood samples were pre-processed differently. To acquire WBCs for neutrophil isolation, RBC lysis buffer (eBioscience) was added into whole blood (10:1 v/v) for 3 min and quenched with 0.5% bovine serum albumin (BSA, Biowest) in phosphate buffered saline (PBS, Lonza). To acquire peripheral blood mononuclear cells (PBMCs) for monocytes or lymphocytes isolation, whole blood was subjected to Ficoll-Paque™ Plus (GE Healthcare) density centrifugation according to manufacturer's protocol. After sample pre-processing, leukocyte subtypes were then sorted using Dean flow fractionation with our previously developed microchip (Hou et al. 2016). Monocytes or neutrophils were collected from outlet 2 while lymphocytes were collected from outlet 3 of the device. The sorted cells were washed and resuspended to 1×10^6 cells/mL in 0.1% BSA for downstream processing.

3.3.2 Monocyte treatment

To study inflammatory status of monocytes for different stimulus and treatment duration, whole blood was treated with tumor necrosis factor- α (TNF- α , 10 ng/mL, Peprotech) or lipopolysaccharides (LPS, 1&10 μ g/mL, Sigma-Aldrich) for 4 h or 8 h at room temperature before monocyte isolation.

3.3.3 Macrophage differentiation from primary monocytes

To differentiate primary monocytes to macrophages, the DFF purified monocytes were first seeded on a 6-well plate and incubated for 24 h at 37°C in RPMI 1640 media (Hyclone) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin (P/S, Life Technologies) for cell adhesion. Following incubation, the non-adherent cells were removed and new media containing macrophage

colony-stimulating factor (M-CSF, 100 $\mu\text{g/mL}$, Miltenyi Biotec) was added to the attached monocytes to induce differentiation (Day 0). The macrophages were sampled on Day 4 and Day 7 for electrical characterization. To harvest the cells, the wells were washed twice with ice-cold $1\times$ PBS and incubated with 2 mM EDTA (Life Technologies) for 15 min at 37°C to detach the macrophages. A cell scraper (Corning®) was used to dislodge the cells gently. The dissociated cells were then washed and resuspended in 0.1% BSA in PBS to a concentration of 1×10^6 cells/mL.

3.3.4 Monocyte subsets sorting

To isolate monocytes subsets, granulocytes (NK, B and T cells) were first depleted from PBMCs with $\alpha\text{-CD15}$, $\alpha\text{-CD56}$, $\alpha\text{-CD19}$ and $\alpha\text{-CD3}$ conjugated microbeads, using magnetic-automated cell sorting (MACs) (Miltenyi Biotec). Monocyte subsets were then identified from the negative fraction using $\alpha\text{-CD14}$ (clone 61D3, Ebioscience), $\alpha\text{-CD16}$ (clone VEP13, Miltenyi Biotec) and $\alpha\text{-CD56}$ (clone NCAM16.2, BD bioscience), and purified by fluorescence-activated cell sorting (FACS) according to the relative CD14 and CD16 expression within the CD56 negative population. The gating strategy for the three monocyte subsets were $\text{CD14}^{++}/\text{CD16-}$ (classical), $\text{CD14}^{++}/\text{CD16}^{+}$ (intermediate) and $\text{CD14}^{+}/\text{CD16}^{++}$ (non-classical).

3.3.5 THP-1 differentiation

Human monocytic cell line THP-1 cell line was maintained in RPMI 1640 media (10% FBS, 1% P/S). The differentiation protocol was adapted from previous literature. Briefly, THP-1 were seeded into a 6-well plate at a concentration of 1×10^6 cells/well and treated with 100 ng/mL phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) for 24 h in an incubator. Following the incubation, the PMA-containing media and non-adherent cells were removed and replaced by fresh RPMI 1640 (10% FBS, 1% P/S).

The cells were allowed to rest for 24 hours (PMAr) and the differentiated macrophages were harvested as previously described.

3.3.6 ox-LDL uptake by THP-1 macrophages

For low density lipoprotein (LDL) uptake study, the fully differentiated and rested macrophages were treated with Human DiI medium oxidized LDL (oxLDL, 10 $\mu\text{g/mL}$, Precision Technologies) for 24 h.

3.3.7 Sample processing for NETosis studies

For NETosis studies, neutrophils in lysed blood were prepared from 200 μL of whole blood (for 5 conditions; before and after control, NETosis at 3 different time points) as aforementioned. After washing for two times, the washed sample were treated with calcium ionophores (2 $\mu\text{g/mL}$) and incubated at 37°C. The neutrophils undergoing NETosis were sampled for impedance measurement at various time points namely 30 min, 60 min and 120 min. Prior impedance characterization, the samples were diluted to 3mL without washing. For glucose treated samples, 30 mM of D-glucose was added to whole blood and the sample was incubated for 4 h at room temperature. Following that, the sample was processed as aforementioned protocol.

3.3.8 Immunofluorescent staining

The DFF-sorted monocytes from T2DM patients were stained 30 min at 4°C with fluorescein isothiocyanate (FITC)-labelled anti-human CD41a antibody for platelets (eBioscience, 1:20 dilution), phycoerythrin (PE)-labelled anti-human CD14 antibody (eBioscience, 1:20 dilution) for monocytes and Hoechst 33342 nucleic acid stain (1 $\mu\text{g/mL}$, Life Technologies) for cell nucleus. The samples were washed once to remove excess antibodies and spotted on a glass slide for imaging. Monocyte-platelet

aggregates (MPAs) were visualized at 100× magnification with fluorescent imaging using Metamorph software (Molecular Devices).

3.3.9 Quantification of NETosis using microplate reader

Neutrophils were seeded on a 96-well clear bottom black plate (BD Biosciences) at a concentration of $\sim 10^5$ cells per well in 200 μ L of media (0.1% BSA in PBS), and stimulated with 20 mM CaI. Unstimulated neutrophils served as negative controls. For each condition, either 500 nM SYTOX green were added to detect DNA release. The progress of NETosis was assessed by measuring the fluorescence of SYTOX Green-DNA interactions (excitation = 485 nm, emission = 528 nm) using a microplate reader (BioTek Synergy H1 Hybride multi-mode reader) every 30 min for 120 min after stimulation. Duplicates for each condition were performed.

3.4 Flow cytometry analysis

For the determination of leukocyte composition, pre-sorted samples (PBMCs, lysed blood and diluted blood) were stained for 30 min at 4°C with allophycocyanin (APC)-labeled anti-human CD66b, PE-labeled anti-human CD14 and FITC-labeled anti-human CD3/CD19 antibodies to identify neutrophils, monocytes and T/ B-lymphocytes respectively. Non-specific antibody binding was examined using corresponding isotype negative control antibodies. All antibodies were purchased from eBioscience (1:20 dilution) and analyzed using a BD LSR Fortessa flow cytometer (BD Biosciences).

3.5 Impedance simulation

To determine the frequency range suitable for impedance measurement, the impedance simulations were performed to study the effect of dielectric properties of cell components on current spectra. As described in Chapter 2, the simulation was based on

Maxwell mixture theory with double shell model (Figure 3.4A) to derived cell impedance and the parameters used for simulation are shown in Table 3.1. For the double shell model, there is nucleus suspending in cytoplasm which are nucleus envelop and nucleoplasm as shown in Figure 3.4B. The calculation of complex permittivity of mixture can be divided into 4 steps.

a. Calculation of complex permittivity of nucleus ($\epsilon_{nucleus}^*$)

$$\epsilon_{nucleus}^* = \epsilon_{np}^* \frac{2(1 - \varphi_2) + (1 + 2\varphi_2) \frac{\epsilon_{ne}^*}{\epsilon_{np}^*}}{(2 + \varphi_2) + (1 - \varphi_2) \frac{\epsilon_{ne}^*}{\epsilon_{np}^*}} \quad (3.10)$$

$$\varphi_2 = \left(\frac{Rne - dne}{Rne} \right)^3$$

Where $\epsilon^* = \epsilon - j \frac{\sigma}{\omega}$ denotes complex permittivity, subscripts “nucleus”, “np” and “ne” represent nucleus, nucleoplasm and nucleus envelop, respectively. and φ_2 is the volume fraction which is the ratio of volume of nucleoplasm to volume of nucleus.

b. Calculation of complex permittivity of cytoplasm contain (ϵ_{cn}^*)

$$\epsilon_{cn}^* = \epsilon_{cp}^* \frac{2(1 - \varphi_1) + (1 + 2\varphi_1) \frac{\epsilon_{nucleus}^*}{\epsilon_{cp}^*}}{(2 + \varphi_1) + (1 - \varphi_1) \frac{\epsilon_{nucleus}^*}{\epsilon_{cp}^*}} \quad (3.11)$$

$$\varphi_1 = \left(\frac{Rne}{R - d} \right)^3$$

Where subscripts “cn”, “cp” and “nucleus” represent cytoplasmic contain, cytoplasm, and nucleus, respectively. and φ_1 is the volume fraction which is the ratio of volume of nucleus to volume of cytoplasm.

c. Calculation of complex permittivity of cell (ϵ_{cell}^*)

$$\varepsilon_{cell}^* = \varepsilon_{mem}^* \frac{2(1 - \varphi_{mc}) + (1 + 2\varphi_{mc}) \frac{\varepsilon_{cn}^*}{\varepsilon_{mem}^*}}{(2 + \varphi_{mc}) + (1 - \varphi_{mc}) \frac{\varepsilon_{cn}^*}{\varepsilon_{mem}^*}} \quad (3.12)$$

$$\varphi_{mc} = \left(\frac{R - d}{R} \right)^3$$

Where subscripts “cn”, “mem” and “cell” represent cytoplasmic contain, membrane, and cell, respectively. and φ_{mc} is the volume fraction which is the ratio of volume of cytoplasm to volume of cell.

d. Calculation of complex permittivity of mixture (ε_{mix}^*)

$$\varepsilon_{mix}^* = \varepsilon_{med}^* \frac{2(1 - \Phi) + (1 + 2\Phi) \frac{\varepsilon_{cell}^*}{\varepsilon_{med}^*}}{(2 + \Phi) + (1 - \Phi) \frac{\varepsilon_{cell}^*}{\varepsilon_{med}^*}} \quad (3.13)$$

$$\Phi = \left(\frac{\frac{4}{3}\pi R^3}{whl} \right)$$

Where subscripts “mix”, “med” and “cell” represent mixture, medium, and cell, respectively. and Φ is the volume fraction which is the ratio of volume of cell (sphere) to volume of detection channel (w – width, h – height and, l - length).

Then the impedance of the mixture containing cell can be calculated from the equation (2.3).

Figure 3.4 Impedance simulation model for leukocytes (A) Schematics of equivalent circuit model of a cell inside detection region (left) double shell model for describing leukocytes (right) (B) Equivalent circuit model of single cell impedance measurement (left) and illustration of a single cell in the detection region

Next, we showed the simulation of the variations of dielectric properties of different cellular components were carried out to determine their effect on the impedance response (Z_{mix}). For this simulation, a cell was assumed to be at the center between the electrode pair and a cell is considered as spherical. It should be noted that the parasitic effect of circuit and the effect of electrical double layer were discarded in the simulation.

3.5.1 The effect of size variation on impedance response

First, the simulation to study the effect of cell size on cell impedance response was performed by varying cell size as shown Figure 3.5 and found that cell size is proportional to impedance response magnitude and cell size contributes to magnitude of whole frequency spectrum (100kHz - 10MHz).

3.5.2 The effect of cell membrane properties variation on impedance response

In electrical equivalent circuit model, a cell membrane is regarded as a capacitor as a thin insulating material (dielectric) sandwiched between two electrolytes (surrounding fluid and cytoplasm). The cell membrane capacitance (C_{mem}) is denoted as follows.

$$C_{mem} = \frac{\epsilon_{mem}\epsilon_0}{d}A \quad (3.14)$$

Where C_{mem} is cell membrane capacitance. ϵ_{mem} is relative permittivity of cell membrane. ϵ_0 is permittivity of free space. A is cell surface area and d is cell membrane thickness.

Figure 3.5 The effect of cell size variation on cell current response

Next, we simulated the effect of variations of cell membrane capacitance on the impedance response by varying permittivity of cell membrane (ϵ_{mem}). It was found that cell membrane capacitance is inversely proportional to impedance response and it greatly impacts impedance response in range of 0.5 MHz – 3 MHz (Figure 3.6A). To mitigate the effect of cell size, several literatures defined opacity as ratio of impedance response or impedance response at two different frequencies (Holmes et al. 2009; van Berkel et al. 2011).

$$Opacity = \frac{|Z_{HF}|}{|Z_{LF}|} \quad (3.15)$$

Figure 3.6 (A) Effect of cell membrane variation on impedance spectra (B) Effect of cell membrane variation on opacity spectra

Where $|Z_{HF}|$ is cell impedance response at high frequency and $|Z_{LF}|$ is cell impedance response at low frequency.

This parameter is commonly used in impedance cytometry to minimize the effect of cell size dependence (Cheung et al. 2005; Gawad et al. 2004). As seen in the simulation, cell membrane capacitance influences impedance response at over 0.5 MHz and cell size dominates impedance spectra at any frequency. Therefore, a division of impedance at high frequency to low frequency is performed to acquire a parameter that represents cell membrane capacitance and minimally depends on cell size. In this work, HF and LF corresponds to 1.72 MHz and 0.3 MHz, respectively. This set of frequencies were used by several literatures. To simulate a dependency of opacity on cell membrane capacitance, we divided the whole frequency spectrum by impedance response at 0.3 MHz (Figure 3.6B) and the similar trend was observed in the opacity when varying the cell membrane capacitance. Thus, to correlate with experimental data in the latter parts of this thesis, simulation plots will be presented as opacity versus frequency onwards.

In biology, changes of cell membrane capacitance can be associated with several factors namely membrane composition and surface morphologies. For mammalian cell, cell membranes consist of a lipid bilayer composed primarily of phospholipids and cholesterol. Different cell types also have different membrane compositions which contribute to differences in cell dielectric properties (ϵ_{mem} , d , A). Surface morphologies such as microvilli, blebs, membrane fold and ruffles, play important roles in the progression of certain cellular functions and they also contribute to cell membrane capacitance through a change in surface area ($C_{mem} \propto A$) (Pethig et al. 2002). For example, when activated-leukocytes undergo a process called apoptosis (programmed cell death), their cell membranes became smoother (reduction of surface area) as

compared to an untreated condition resulting in the reduction in cell membrane capacitance (Pethig et al. 2002; Pethig and Talary 2007; Wang et al. 2002).

3.5.3 The effect of cytoplasm properties variation

Next, we simulated the effect of variations of cytoplasm conductivity on the impedance response as shown in Figure 3.7. As a result, it was observed that an increase in cytoplasm conductivity significantly contributes to an increase in impedance response at high frequency ($>2\text{MHz}$). The reason why the cytoplasm contribution on impedance response become apparent at high frequency is possibly because the cell membrane capacitance is capacitively short circuited by its reactance at high frequency (inversely proportional to applied frequency) thus enabling a manifestation of cytoplasm properties.

Certain cell activities have been reported to affect cytoplasm properties. For example, when Phorbol 12-Myristate 13-Acetate (PMA) activated neutrophils undergo a formation of neutrophil extracellular traps (NETosis), there was an increase or decrease in cytoplasm conductivity due to massive Ca^{2+} influx (Griffith and Cooper 1998; Kenny et al. 2017).

Figure 3.7 Effect of cytoplasm variation on opacity spectra

Figure 3.8 The effect of nucleus size variation on opacity spectra

3.5.4 The effect of nucleus size variation

The effect of nucleus size (R_{ne}) variation on opacity was examined as shown in Figure 3.8. By increasing nucleus size, we observed an increase in opacity at high frequency (>3 MHz). The minimal change at low frequency may probably be hindered by the cell membrane capacitance as it prevents a current flow into a cell (large impedance at low frequency).

3.5.5 The effect of different models

Under certain cellular functions (e.g. NETosis), biological cells may undergo structural changes resulted in a loss of nucleus which can be modeled as a change from a double shell model to a single shell model. In this section, we compared opacity response of different models namely particle without shell (polymer beads), single shell model (a cell without nucleus) and double shell model (a cell with nucleus). As shown in Figure 3.9, a particle without shell exhibits constant opacity over wide range of frequency. This is due to the fact that a particle without shell is an insulating sphere (purely resistive load and frequency independent). Minimal difference in opacity responses from single shell model and double shell model below 2 MHz is found while a significant increase in opacity response from single cell model can be observed at

Figure 3.9 Effect of different model on opacity spectra

frequencies of 2 MHz and above. This can be attributed to the presence of nucleus structure or other intracellular organelles which could also cause additional interfacial polarization (eventually resulted in capacitively short-circuit of nucleus envelop at high frequency.).

Table 3.1 Simulation parameters

Detection region dimension	
Width of the channel, width of the electrode	30 μm
Height of the channel	20 μm
Separation gap between electrodes pair	20 μm
Cell parameters (taken from Griffith and Cooper (1998))	
Cell radius (R)	6 μm
Nucleus radius (R _{ne})	3.5 μm
Thickness of cell membrane (d)	8 nm
Thickness of nucleus envelope (d _{ne})	20 nm
Permittivity of cell membrane (ϵ_{mem})	6.2 ϵ_0
Conductivity of cell membrane (σ_{mem})	10^{-11} S/m
Permittivity of cytoplasm (ϵ_{cp})	60 ϵ_0
Conductivity of cytoplasm (σ_{cp})	0.3 S/m
Permittivity of nucleus envelope (ϵ_{ne})	28 ϵ_0
Conductivity of nucleus envelope (σ_{ne})	6×10^{-3} S/m
Permittivity of nucleoplasm (ϵ_{np})	52 ϵ_0
Conductivity of nucleoplasm (σ_{np})	1.35 S/m
Medium parameters	
Permittivity of medium	80 ϵ_0

Conductivity of medium	1.6 S/m
Constant	
Permittivity of free space (ϵ_0)	10^{-12} F/m

3.6 Impedance measurement setup

Rather than perform impedance measurement, in our experimental setup, we measured a current response from the coplanar electrodes by providing an excitation voltage to the middle electrode while differentially measuring current responses from side electrodes to establish a differential current measurement (Figure 3.10A). The experimental setup will explain later in the impedance measurement setup in this chapter. This measurement scheme can provide a single cell impedance measurement directly against its surrounding medium. Moreover, it also provides 1) baseline subtraction leading to better signal sensitivity (analog to digital convertor can quantize signal in a higher resolution.) 2) reduction of common mode noises such as signal drifting, thermal fluctuation (any noises that appear in both channels (current responses from side electrodes) will be cancel out.). The differential current response when there is a single cell in between one of the side electrodes (another side electrode has no cell.) and the center electrode can be defined as follows.

$$I_{diff}^* = I_{mix}^* - I_{med}^* \quad (3.16)$$

Where I_{diff}^* is the differential current response for the measurement. I_{mix}^* is the current response of mixture (a suspended particle/cell in detection region) and I_{med}^* is the current response of medium within detection region (without any cell).

To establish electrical measurement, the middle electrode was supplied with 2.5-3V at two different frequencies (0.3MHz and 1.72MHz) by a lock-in amplifier (HF2LI, Zurich instrument). When a single cell passes through the electrodes, differential impedance responses were acquired from the side electrodes by using transimpedance

Figure 3.10 Experimental setup: (A) Schematic of experimental setup illustration of microfluidic chips, (B) optical image illustrating single cell flowing through the electrodes in the detection region and (C) Time domain measurement of multiple events from single cells

amplifiers (DHPCA-100, FEMTO®) with transimpedance gain of 10kV/A. Then output signals were sent to the lock-in amplifier to extract a magnitude and phase of the signal of interest at sampling rates of 112k sample/s and 230k sample/s for the combinatorial strategy and the integrated platform respectively (Figure 3.10A). The recorded sequential data in Figure 3.10 was quantified to extract to peaks corresponding to single cell events using a custom-made program written in MATLAB (MathWorks). As a result, impedance magnitude at 1.72MHz and 0.3MHz were acquired.

3.7 Data presentation and statistical analysis

2D scatter plots were based on cell size ($|Z_{LF}|$) and opacity ($|Z_{HF}|/|Z_{LF}|$, ratio of impedance magnitude at 1.72MHz to 0.3MHz). However, for the integrated platform section, the data was presented in term of cell size in μm instead of $|Z_{LF}|$. The following

equation was used to convert impedance magnitude at 0.3MHz ($|Z_{LF}|$) to electrical cell size (De Ninno et al. 2017).

$$Cell\ size = G|Z_{LF}|^{\frac{1}{3}} \quad (3.17)$$

Where G is a gain factor considering electronic setup. This parameter was calculated using impedance parameters from monodisperse beads (reference).

Then 2D scatter plots were based on cell size (μm) and opacity ($|I_{HF}|/|I_{LF}|$, ratio of impedance magnitude at 1.72MHz to 0.3MHz). To take in account of density of cell population, the scatter plots were plotted in MATLAB using dscatter function from MATLAB Central File Exchange (Henson 2016). To simplify data quantification of an impedance profile, density-based spatial clustering of applications with noise (DBSCAN) was used to extract a cell cluster with larger cell size.

Mann-Whitney test was used to determine the statistical significance of the difference between two sets of data. $P < 0.05$ was considered of statistically significant difference. All analysis was performed using GraphPad Prism V7.0 (GraphPad software).

3.8 Study approval

Written informed consent was obtained for all subjects during recruitment. All protocols were approved by the institutional review board of Nanyang Technological University (IRB-2014-04-27), Tan Tock Seng Hospital (2014/00416) and Singapore General Hospital (CIRB2017/2512), in compliance with the Human Biomedical Research Act (Ministry of Health, Singapore). Blood samples were collected either from venepuncture into a 4 mL K2 EDTA tube (BD vacutainer) of healthy and diabetics for DFF isolation or from healthy donors undergoing leukapheresis for monocyte subsets isolation.

3.9 Summary

In summary, this chapter presents the device design principle as well as protocols for experiments in the later chapters. Moreover, it also shows the impedance simulation which can be correlated with experiment results in the later chapters. In low frequency regime (< 1 MHz), impedance response can provide cell size whereas, impedance response at higher frequency regime (>1 MHz) provide information about cell components such as cell membrane, cytoplasm and nucleus. Then we investigated the effect of variation of cell component' dielectric properties on opacity which was defined as ratio of impedance at high frequency to low frequency (0.3 MHz) to minimize cell size dependency. As a result, we observed that opacity at frequency range between 1 and 2 MHz was influenced by cell membrane properties as a function of permittivity of cell membrane (ϵ_{mem}), membrane thickness (d) and surface area of cell (A) which depends on surface morphology. At frequency beyond 2 MHz, opacity conveys information regarding internal components such as cytoplasm, nucleus size because cell membrane capacitance was capacitively short circuited. At this frequency regime, opacity can also distinguish single shell model from double shell model. This is due to the presence of nucleus envelop. However, the inner components are difficult to probe at high frequency measurement, and the measurement suffers from sensitivity and noise issues. As a consequence, we chose 0.3MHz and 1.72MHz as excitation frequencies for the measurement in this work which have been in literatures (Hassan et al. 2015; Hassan et al. 2016).

CHAPTER 4 COMBINATORIAL MICROFLUIDIC STRATEGY FOR LABEL-FREE LEUKOCYTE SORTING AND IMPEDANCE-BASED PROFILING

4.1 Background

Circulating leukocytes are key components of our immune system against infections and are involved in the pathogenesis of major diseases such as cancer (Rüegg 2006), metabolic disorders including diabetes (Gordon and Taylor 2005; Sprangers et al. 2016), and neurological diseases (Wilson et al. 2010). The study of leukocyte heterogeneity and functions is becoming increasingly important in cell biology research to elucidate disease pathobiology and mechanisms (Beyrau et al. 2012), and interrogation of single-cell biophysical and functional phenotype can also serve as intrinsic biomarkers for cell-based diagnostics and therapeutics (Hou et al. 2016; Tay et al. 2018).

As previously mentioned in review chapter, the gold standard for single-cell studies is flow cytometry which enables multi-parametric cell discrimination based on monoclonal antibodies labelling and immunofluorescence at different wavelengths. Impedance cytometry is an attractive alternative for high throughput and label-free single cell analysis based on cell size and intrinsic dielectric properties of cellular components such cell membrane and cytoplasm (Chen et al. 2015; Cheung et al. 2010; Petchakup et al. 2017; Sun and Morgan 2010). The technology has been widely exploited for cell type identification (Hassan et al. 2017; Simon et al. 2016; Spencer et al. 2014a), platelet activation testing (Evander et al. 2013), cancer cell discrimination (Spencer et al. 2014b), as well as detection of parasite-infected cells for disease diagnostics (Du et al. 2013; Küttel et al. 2007). However, electrical characterization of

circulating leukocytes in whole blood remains a technical challenge as conventional leukocyte isolation methods require affinity-based antibodies binding steps which will attenuate membrane properties (Fukuda and Schmid-Schönbein 2002; Jensen 2012). Rollo *et al.* have recently developed an impedance sensor for activated T lymphocytes but it requires negative magnetic selection of target cells (using antibodies) which is expensive and time-consuming. Hence there exists a critical need to develop microfluidic label-free leukocyte sorting technologies with minimal perturbation and manipulation and can be easily combined with impedance cytometry to study leukocytes in their native state. This strategy is more cost-effective and can greatly facilitate automation and assay robustness with minimal user sample manipulation for point-of-care testing.

In this work, a novel microfluidic strategy for rapid and label-free leukocyte sorting and impedance profiling from whole blood or peripheral blood mononuclear cells (PBMCs) is proposed. Leukocytes were size-fractionated into different subtypes (neutrophils, monocytes, lymphocytes) using Dean Flow Fractionation (DFF), a inertial microfluidics cell separation technique previously developed by our group (Hou et al. 2016). Enriched leukocytes were simultaneously washed (Yeo et al. 2015) and eluted off-chip for impedance measurement in a microfluidic impedance flow cytometer with coplanar electrodes. Based on cell size (impedance magnitude at 0.3MHz) and opacity (ratio of impedance magnitude at 1.72MHz to 0.3MHz) which corresponds to cell membrane property (Holmes et al. 2009; van Berkel et al. 2011), the developed platform enabled identification of different leukocyte subtypes after DFF which selectively enriched neutrophils or monocytes from lysed blood or PBMCs respectively. The developed workflow was demonstrated with various utilities namely, activation

profiling and diabetes testing, monocyte differentiation characterization, and cancer cell identification.

4.1.1 Activation profiling and diabetes profiling

Monocytes are highly heterogeneous leukocyte in our body and play a crucial role in pathogenesis of various diseases such as diabetes mellitus. In type 2 diabetes mellitus (T2DM), high blood glucose level (hyperglycemia) can induced monocyte activation (Boden and Rao 2006; Shanmugam et al. 2003). Additionally, it also has been reported that there were a change in number of monocytes (Min et al. 2012) and upregulation of certain inflammatory stimulus such as TNF- α (Mirza et al. 2012) and LPS (Trøseid et al. 2013). In conventional biology, researchers concern about an expression of certain antigens or markers on monocyte surface. However, the studies of markers by FACs require antibody tagging which is time consuming and laborious. There is a critical lack in studies of monocyte morphology and biophysical properties (size, dielectric) which could be potentially exploited for label-free diagnostics or studies. In this work, we hypothesized that inflammatory stimulus could attenuate some changes on biophysical properties of monocytes. Hence, this led us to study the effect of those inflammatory stimulus on monocyte impedance profiles (size and cell membrane properties) using combinatorial strategy. In addition, we also investigated impedance profiles of DFF-isolated neutrophils and monocytes obtained from patients with type 2 diabetes mellitus (T2DM, n=8) to explore the potential of the developed strategy in point-of-care testing.

4.1.2 Monocyte differentiation characterization

Macrophages are responsible for various immune functions such as homeostasis, tissue repair and defence against pathogens (Wynn et al. 2013) and they can be acquired from monocyte differentiation via macrophage-colony stimulating factor (M-CSF). In this part, we sought to determine whether impedance cytometry can electrically probe

monocyte differentiation process. This could provide an alternative to FACs for rapid and label-free determination of monocyte differentiation stages.

4.1.3 Cancer cell identification

Blood cancers represents a group of diseases characterised by interruption of patient's blood cell development by a growth of abnormal/cancerous blood cells. To examine a translational potential of impedance cytometry for blood cancer detection, THP-1 and monocytes were compared. THP-1 are the immortalized monocyte-like cell line derived from a leukemia patient which are commonly used as a model for human monocyte (instead of primary monocytes from blood) (Bosshart and Heinzelmann 2016) and, it has been reported that THP-1 and primary monocytes are different in morphology (Daigneault et al. 2010). The comparative study was carried out to see if impedance cytometry can discriminate healthy cells from their cancerous counterpart.

4.2 Experimental section

4.2.1 Leukocyte impedance measurement

Prior to sample perfusion into the device, the device was flushed with 70% ethanol followed by deionized water and primed with sheath (0.1% BSA in PBS) for 30 min to prevent non-specific binding. 5 μ m polystyrene beads (Nalgene) were added into the sample as an internal reference. The experimental setup and schematic of microfluidic device is shown in Figure 4.1B. The sample and sheath solution were loaded into 1 mL syringes and perfused into the device at 5 μ L/min using syringe pumps (Chemyx). The device was allowed to run for 1 min to stabilize the flow prior to electrical measurement.

4.3 Results and discussion

4.3.1 Impedance-based leukocyte subtypes identification

To determine if DFF leukocyte isolation step can enhance impedance detection, the impedance profile of different blood cell samples was first characterized, namely the diluted whole blood, peripheral blood mononuclear cells (PBMCs), DFF-sorted monocytes, DFF-sorted lymphocytes and DFF-sorted neutrophils. Single-cell impedance measurements were plotted as a 2D scatter distribution plot based on cell size ($|Z_{LF}|$, impedance magnitude at 0.3MHz) and opacity ($|Z_{HF}|/|Z_{LF}|$, ratio of impedance magnitude at 1.72MHz to 0.3MHz) which corresponds to cell membrane property (Holmes et al. 2009; van Berkel et al. 2011). As shown in Figure 4.2A, diluted whole blood sample had low impedance signal of $|Z_{LF}|$ below 0.002 V which comprised mainly of RBCs and platelets as these are the most abundant cells (~100-1000-fold more than leukocytes) in whole blood. The PBMCs impedance profile was significantly different from whole blood with the depletion of RBCs. To facilitate analysis, the impedance profiles were divided into 3 zones based on cell size to distinguish RBCs and platelets (zone 1, RBCs: 5–6 μ m; platelets: 2–3 μ m), lymphocytes (zone 2, 7–8 μ m) and neutrophils/monocytes (zone 3, 10–15 μ m). As expected, PBMC samples consisted of ~80–85% lymphocytes and ~15–20% monocytes, which corresponded to the presence of a large and small population in zones 2 and 3, respectively. There was also a

Figure 4.1 Workflow of leukocyte impedance phenotyping strategies starting with sample pre-processing (RBC lysis or Ficoll density centrifugation), DFF leukocyte sorting and impedance profiling

significant population in zone 1 which were the platelets as these are usually isolated in the PBMCs fraction during Ficoll centrifugation. The impedance gating strategy was further validated on DFF-sorted lymphocytes and monocytes showing observed similar distribution patterns in zone 2 and 3 respectively. Impedance signal was clearly enhanced after DFF enrichment, and lymphocyte opacity (~ 1.2) was higher than monocytes (~ 1.1) in both PBMCs and DFF-sorted samples, indicating distinct differences in the membrane properties of these 2 cell types. The DFF-sorted neutrophils

Figure 4.2 Impedance profiling of different blood cell samples (A) Impedance profiles (density scatter plot of cell size ($|Z_{LF}|$, V) versus opacity) of diluted whole blood (1000 $\times$ dilution), PBMCs, DFF-sorted monocytes, DFF-sorted lymphocytes and DFF-sorted neutrophils. Impedance profile was gated into 3 different zones based on cell size to distinguish cellular debris, platelets and RBCs (zone 1: 0.0004-0.002V), lymphocytes (zone 2: 0.002-0.007V), and monocytes and neutrophils (zone 3: 0.007-0.015V). Due to high density of zone 1 population, the scatter density of zone 1 was calculated separately from zone 2 and zone 3. (B) Frequency distribution of different leukocyte subtypes in each zone for unsorted PBMCs and DFF-sorted leukocytes to determine cell purity.

were characterized, and it was found that they were residing in zone 3 due to their similarities in cell size with monocytes. Of note, there was a significant depletion of platelets in zone 1 for DFF-sorted samples due to the buffer exchange capabilities of DFF which enables leukocytes to be eluted in new buffer prior to analysis (Yeo et al. 2015). This is an important feature as the presence of small particles (such as a platelet or cell debris) can affect impedance measurement when pass through the detection region simultaneously. Neutrophils had a higher opacity (~ 1.2) than monocytes (~ 1.1) and was similar to previous work (Holmes et al. 2009). For analysis of cell purity after DFF, we measured the frequency distribution of the various leukocyte subtypes of unsorted PBMCs and DFF-sorted samples (Figure 4.2B). The different leukocyte subtypes were sufficiently enriched ($\sim 60\text{--}80\%$) for downstream impedance detection. Taken together, these results clearly demonstrate the capabilities and importance of label-free DFF isolation to obtain enriched leukocyte subtypes for enhanced impedance measurement detection sensitivity and selectivity.

4.3.2 Impedance-based leukocyte activation profiling

The effects of inflammatory stimulus (TNF- α and LPS) on the impedance profile of DFF-sorted monocytes were investigated. PBMCs were first isolated and monocytes were then sorted using DFF after treatment of whole blood samples with TNF- α (10ng/mL) or LPS (10 μ g/mL) for 4 h. Enriched monocytes were then perfused through the developed microfluidic impedance device and ~1000–5000 single cell events were recorded (~5 min) for analysis. As shown on Figure 4.3A, the impedance scatter distribution was higher for TNF- α -treated monocytes as compared to untreated

Figure 4.3 Impedance-based activation profiling of enriched monocytes (A) Representative impedance profile of untreated (left), TNF- α treated (center) and LPS treated monocytes (right) with number of cells (n) (B) Average monocyte opacity (left) and cell size (right) for different conditions. Values were taken from the centroid positions of impedance profile for untreated, TNF- α , LPS_{low} (black circle) and LPS_{High} (green circle). Bars were represented as mean $\pm$ s.e.m. *P $\leq$ 0.05, **P $\leq$ 0.01 (C) Flow cytometry analysis of leukocyte composition of PBMCs and DFF-sorted samples (n=4). (D) Flow cytometry analysis of monocyte activation markers CD14 (top) and CD11b (bottom) at different conditions.

monocytes obtained from the same individual. Interestingly, two distinct subpopulations were observed for LPS-treated monocytes which were defined as LPS_{low} (equivalent opacity and size distribution) and LPS_{High} (higher opacity and smaller cell size) as compared to untreated monocytes. We further quantified the mean opacity and cell size based on the centroid of the impedance distribution. Opacity was significantly higher for both TNF α -treated and LPS_{High} monocytes ($P < 0.01$) as compared to untreated monocytes, and there were negligible differences in cell size among all groups except between LPS_{low} and LPS_{High} monocytes ($P < 0.05$) (Figure 4.2B). It was sought to determine the leukocyte composition of the treated blood samples before and after DFF sorting using flow cytometry. Increased neutrophil population (CD66b+) were found in the PBMCs of both TNF- α and LPS treated blood (TNF- α ~28%, LPS ~17% and untreated ~3%), which was likely due to low-density neutrophils that had undergone degranulation during activation (Deng et al. 2016; Nicolás-Ávila et al. 2017). Their presence in PBMCs were also reported in different diseases (Carmona-Rivera and Kaplan 2013; Deng et al. 2016; Fu et al. 2014; Herteman et al. 2017) (Figure 4.3C). While previous literatures have reported similar impedance between monocytes and neutrophils (Hassan et al. 2016; Holmes and Morgan 2010; Holmes et al. 2009; Simon et al. 2016), this set of results indicated the LPS-treated samples comprised of two populations namely LPS_{High} (neutrophils) and LPS_{low} (monocytes) as evident in Figure 4.3A. It was further investigated the effects of LPS treatments (concentration and duration) and found that LPS_{high} and CD66b+ populations increased in a time and dose-dependent manner in both impedance measurements and flow cytometry analysis, thus confirming that the LPS_{High} population consists of neutrophils (Figure 4.4). Noteworthy, flow cytometry analysis of TNF α -treated samples revealed higher neutrophil population (~28%), but there was only a single population distribution in our impedance

measurement as opposed to LPS treatment. It is hypothesized that TNF- α might induce significant changes on monocyte membrane properties which resulted in similar impedance characteristics similar to low-density neutrophils (shift towards higher opacity and lower cell size). The monocyte membrane protein expression was further characterized for CD14 and CD11b, which were related to monocyte activation status (Abeles et al. 2012; van Oostrom et al. 2004) (Figure 4.3D), and observed significant upregulation of both markers for TNF α and LPS treated conditions as compared to untreated monocytes. Overall, these results suggest the use of impedance measurements

Figure 4.4 The effect of LPS concentration and duration variation on impedance profile of sorted leukocytes (A) Impedance profiles of different conditions namely control sample (healthy monocytes), sample treated with low concentration of LPS for 4 hours, sample treated with LPS for 4 hours and sample treated with LPS for 8 hours; Blue and red boundary are used to highlight LPS_{high} and LPS_{low} respectively. (B) Flow cytometry analysis of leukocyte composition of control and LPS treated monocytes at different durations and doses. (n=2)

to characterize leukocytes activation and monitor membrane-associated cellular processes. The proposed strategy can also be used as a novel and label-free approach to detect low-density neutrophils for point-of-care testing.

4.3.3 Impedance-property of monocyte differentiation into macrophages

Monocytes differentiation into macrophages is a vital immune function and they are responsible for homeostasis, tissue repair and defence against pathogens (Wynn et al. 2013). To determine if impedance signal can be used to monitor the differentiation process, we measured monocytes impedance at day 0 (undifferentiated), day 4 (partially differentiated) and day 7 (fully differentiated) when treated with M-CSF to induce macrophages formation (Figure 4.5A). Representative impedance profiles of monocytes

Figure 4.5 Characterization of monocyte differentiation into macrophages and monocyte subsets characterization (A) 20× Brightfield images of seeded monocytes (day 0), and macrophages after 4 days 7 days of differentiation with M-CSF. Scale bar = 20 μ m (B) Impedance profile of monocytes, monocytes undergoing differentiation at day 4, and fully-differentiated macrophages at day 7 (MØ). (C) Average monocyte size (bottom) and opacity (top) at different differentiation stages. Bars were represented as mean $\pm$ s.e.m. *P < 0.05, **P < 0.01

at different differentiation stages were shown in Figure 4.5B. Impedance distribution was clearly more heterogeneous and widespread during the process. As monocytes undergo differentiation, we observed a significant increase in cell size at day 4 ($P < 0.05$) and day 7 ($P < 0.01$) as compared to day 0. (Figure 4.5C). Average opacity was similar at day 4 but decreased slightly at day 7 (no significant differences) probably due to the increase in surface area. These results suggest the feasibility of using impedance cytometry as a label-free approach to monitor monocyte differentiation.

4.3.4 Monocyte subset characterization

Monocyte is a heterogeneous cell population and can be classified into classical ($CD14^{++}/CD16^{-}$), intermediate ($CD14^{++}/CD16^{+}$) and non-classical ($CD14^{+}/CD16^{++}$) monocytes based on CD14 and CD16 expressions (Boyette et al. 2017; Wong et al. 2011). Previous literatures have reported the role of different monocyte subsets in the pathogenesis of cardiovascular diseases (Shantsila et al. 2011; Wildgruber et al. 2016), and identification of specific monocyte subsets would be imperative for functional studies and clinical testing. In this study, the impedance profiles of purified monocyte subsets pre-sorted using FACS were characterized. Both classical and non-classical monocytes exhibited a single-peak profile while intermediate monocytes had a relatively more heterogeneous impedance distribution (Figure 4.6A). Figure 4.6C shows the normalized opacity and size of each monocyte subset from 4 healthy subjects. Intermediate monocytes were found to be the largest in cell size and non-classical monocytes were the smallest, which were consistent with prior literatures (Cros et al. 2010; Shantsila et al. 2011). Interestingly, intermediate monocytes had the lowest opacity as compared to classical monocytes ($P < 0.01$), and future investigations are warranted to study the associations between monocyte dielectric properties and their functional phenotypes. It should be noted that monocyte subsets were sorted based on

antibodies labelling that can inherently affect their cellular functions and properties (Jensen 2012; Progatzy et al. 2013). In our experiments, we also observed subtle differences in impedance profile between unstained and stained monocytes (Figure 4.6D), which further highlight the importance of antibodies-free monocyte isolation for

Figure 4.6 Characterization of monocyte subsets characterization (A) Representative impedance profiles of classical (left), intermediate (center) and non-classical monocytes (right) (B) Raw data for opacity and cell size before normalization from 4 different subjects (C) Normalized opacity (left) and size (right) for monocyte subtypes from 4 different subjects. The normalization was acquired by dividing opacity of each subset by the average value of every subsets (same subject). Paired t-test was used to determine the statistical significance of the difference between two sets of data. *P < 0.05, **P < 0.01. (D) Impedance profiles of 1. unstained monocytes 2. stained monocytes (before FACs sorting)

impedance-based assays. Nevertheless, our data clearly demonstrated subtle changes in impedance characteristics of different monocyte subsets which can be further exploited for label-free cell sorting.

4.3.5 Impedance-based immune health profiling in type 2 diabetes mellitus (T2DM)

As proof-of-concept for rapid and label-free immune health profiling, the dielectric properties of neutrophils and monocytes isolated by DFF from T2DM patients (n=8, Table 4.1) were measured. T2DM was chosen as the disease model because neutrophils and monocytes are widely implicated in the disease pathogenesis (Giulietti et al. 2007; Min et al. 2012), and have been proposed as biomarkers for atherosclerosis risks in T2DM (Patkó et al. 2012). Figure 4.7A shows the impedance-based characterization of cell size and opacity of monocytes and neutrophils in T2DM patients. It was not obvious of any associations between leukocyte size and opacity (Figure 4.9A, B). While there were negligible differences in $|Z_{LF}|$ (cell size) of both populations, neutrophil opacity was significantly higher than monocytes ($P < 0.05$), and neutrophil and monocyte opacity was strongly correlated for all subjects (Figure 4.7B). In general, opacity and size were higher in diabetic monocytes as compared to healthy controls (Figure 4.7C). This trend is indeed consistent with *in vitro* TNF- α treated monocytes, thus suggesting a pro-inflammatory signature in T2DM subjects. Monocyte-platelet aggregates (MPA) was also present in DFF-sorted monocytes based on immunofluorescence staining for platelets (CD41a), monocytes (CD14) and cell nucleus (Hoechst dye) (Figure 4.7D). This is interesting as MPA is a sensitive indicator for platelet activation (Michelson et al. 2001) and can alter the monocytes towards a more atherogenic phenotype (Linden and Jackson 2010). Moreover, platelet binding to monocyte surface can also contribute to biophysical changes in monocyte size ($|Z_{LF}|$) and opacity.

To further understand the clinical significance of the leukocyte impedance measurements using the developed technology, it is necessary to determine their associations with established cardiovascular risk factors (blood pressure, lipids, glycaemic, CRP *etc.*) and vascular functions. The results showed that diabetic monocyte size ($|Z_{LF}|$) was associated with increasing non high-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol levels ($P < 0.05$) (Figure 4.8A,B), which is important as LDL cholesterol accelerates monocyte-macrophage differentiation (Escate et al. 2016) and play a key role in lipid loading in monocytes (Fernandez-Ruiz et al.

Figure 4.7 Impedance-based immune health profiling in type 2 diabetes mellitus (T2DM) (A) Measurement of cell size and opacity of monocytes and neutrophils in T2DM patients. (B) Strong correlation of monocyte and neutrophil opacity in T2DM patients. (C) Scatter plot of monocyte impedance phenotype in T2DM patients ($n = 8$). Blue shaded box indicates impedance profile distribution of non age-matched healthy monocytes ($n=4$) and is plotted based on geometrical mean ± 2 S.D. Blue dot represents an age-matched healthy subject which resides within the healthy zone. (D) Representative brightfield and immunofluorescence images of T2DM monocytes with the presence of monocyte-platelet aggregates (MPAs) after DFF.

2016). Similar trends were also observed for total cholesterol ($P=0.06$) and triglyceride levels ($P=0.08$) (Figure 4.9D, E). Monocyte opacity decreased with reactive hyperemia index (LnRHI) ($P < 0.05$) as shown in Figure 4.8C, a measurement of endothelial function and a powerful surrogate marker for cardiovascular health (Rubinshtein et al. 2010). With a cutoff of <0.51 defined as abnormal or dysfunctional endothelium, this corresponded to T2DM subjects with the highest monocyte opacity (most activated). It was also observed decreasing monocyte opacity with hyperlipidemia duration ($P < 0.05$) (Figure 4.8D). A possible reason is that T2DM subjects with hyperlipidemia are taking lipid-lowering medications (e.g. statins), and subjects with more recent hyperlipidemia (thus less medication) may still reflect an overall more activated monocyte phenotype. Lastly, neutrophil size and opacity with cardiovascular risk factors were also characterized. While it is not obvious of any associations with lipid levels, neutrophil size was strongly correlated to C-reactive protein (CRP), a key marker for low-grade inflammatory state and cardiovascular risk ($P < 0.05$) (Figure 4.8E) and duration of

Figure 4.8 Correlation of monocyte and neutrophil impedance parameters with (A) Non-HDL cholesterol, (B) LDL Cholesterol, (C) LnRHI, (D) Hyperlipidemia and correlation of neutrophil impedance parameters with (E) C-reactive protein (CRP) and (F) Hyperlipidemia

hyperlipidemia ($P < 0.05$) (Figure 4.8F). Taken together, these results strongly support our hypothesis that T2DM subjects with severe endothelial dysfunction or higher cardiovascular risks have a more pro-inflammatory (more activated) neutrophil/monocyte phenotype as seen by their higher size and opacity. However, the sample size in this study is small, and a large-scale clinical validation is necessary to confirm our hypotheses and further develop these impedance-based biomarkers for label-free immune health profiling.

Figure 4.9 Correlation of monocyte and neutrophil impedance parameters with (A) Correlations of monocyte size with neutrophil size, correlations between average opacity and cell size from monocyte (B) and neutrophil (C) and correlations of average monocyte size with other clinical relevant parameters such as (D) total cholesterol and (E) triglyceride

Table 4.1 Clinical demographics of T2DM patients

Characteristics	T2DM (n = 8)
Age* (Range)	59 (47 - 64)
BP (Sys)	132.188 (3.537)
BMI	27.343 (1.929)
CRP, mg/L	1.263 (0.480)
HbA1c, %	8.338 (0.554)
Fasting Glucose	7.700 (0.825)
Total-C	3.838 (0.413)
HDL-C	1.025 (0.059)
Non-HDL-C	2.813 (0.402)
LDL-C	2.077 (0.232)
Triglyceride	1.875 (0.590)

Average value shown with SEM in parentheses, unless otherwise indicated.

4.3.6 Monocytes and cancerous monocytic cells comparison

Generally, it is known that the cell lines or cancerous cells are larger than their primary counterpart (Becker et al. 1995; Spencer et al. 2014b). In this part, a comparison was made on the impedance profile of primary monocytes to their widely used immortalized monocytic cell lines called (THP-1) (Qin 2012). Overlaid impedance profiles of both populations show distinct size difference between primary monocytes and THP-1 (Figure 4.10A) indicating discrimination of monocytes from their cancerous counterpart.

4.3.7 ox-LDL uptake by THP-1 macrophages

Monocyte LDL uptake is a key process in atherosclerosis to form foam cells and the development of atherosclerosis lesions. To study cellular LDL uptake using the developed impedance cytometry, LDL internalization in THP-1-differentiated macrophages was characterized. The microscopic images of cultured THP-1 macrophages with and without LDL treatment are shown in Figure 4.10B. It was observed that found that there was an increase in cells with lower opacity and larger cell size as compared to control sample (38% for LDL sample vs. 23% for control sample based on our gating strategy highlighted in black dotted box in Figure 4.10B (bottom)).

Figure 4.10 Other applications (A) Discrimination of cancer cells (B) Characterization of ox-LDL uptake by THP-1 macrophages; Black dotted line represents threshold for LDL uptake determination. (Right) Microscopic images of control (top) and LDL sample (bottom). Red color represents dyed LDL internalized by the cell.

A possible explanation for the observed impedance changes was that cells they became larger after LDL uptake and morphological changes on the membrane (e.g. smoother membrane) will lead to a decrease in opacity.

Overall, the results show the versatility of developed platform in leukocyte impedance phenotyping using coplanar electrode configuration. Although a parallel electrode configuration has a better sensitivity and detection limit as compared to the coplanar electrode configuration (adopted in this work) owing to less divergent electric field (Sun et al. 2008), the fabrication process is more complicated and more expensive. Herein, we demonstrated that the subtle changes in dielectric properties of cells can be detected even with coplanar electrode configuration after a microfluidic purification step. Additionally, the coplanar electrode configuration facilitates integration with other

microfluidic modules for “sample in-answer out” platforms. In term of response time, there are minimal differences between parallel and coplanar electrode designs since they both require similar electronic setups (transimpedance amplifiers and lock-in amplifier). Lastly, the operating throughput of our developed cytometer is in the range of 400-1000 cells/min, which is comparable to systems from other groups (Cheung et al. 2005; Rollo et al. 2017; Zhou et al. 2018).

4.4 Summary

In summary, a novel microfluidic strategy for rapid and label-free leukocyte sorting coupled with impedance profiling from whole blood was developed. It was demonstrated that it enhanced impedance detection selectivity when DFF was used to fractionate and enrich for different leukocyte subtypes prior to analysis in a microfabricated impedance cytometer. The impedance-based monocyte quantification was successfully used for monocyte activation profiling, monitoring monocytes differentiation to macrophages, as well as biophysical characterization of monocyte subsets (classical, intermediate, non-classical). We also clinically validated the developed technology for label-free leukocyte impedance profiling in T2DM subjects where we observed that the leukocyte dielectric properties (size and opacity) were significantly associated with cardiovascular risk factors including lipid levels, C-reactive protein (CRP) and vascular function (LnRHI). It is envisaged that the proposed strategy will greatly facilitate immunology research, and future work includes the integration of leukocyte DFF sorting module with impedance cytometry on a single chip for rapid point-of-care testing.

CHAPTER 5 INTEGRATED PLATFORM FOR LEUKOCYTE SORTING AND IMPEDANCE PHENOTYPING

5.1 Background

In Chapter 4, the combinatorial microfluidic strategy for leukocyte phenotyping by enriching target leukocyte subtypes (neutrophils and monocytes) by DFF prior impedance cytometry was shown and found to increase the detection selectivity. Although impedance cytometry is capable of identifying every blood component, phenotyping of specific subtypes from unprocessed samples (diluted blood/PBMCs) will require a long measurement session to acquire statistical meaningful data. This strongly advocates a realization of DFF sorter and impedance cytometer on a single chip to facilitate clinical applications. However, the integration is non-trivial task since there exists flow rate disparity between DFF and impedance cytometer. By accounting for flow rate mismatch between DFF (Hou et al. 2016) sorter and impedance cytometer using particle inertial focusing effects in serpentine channels (Di Carlo et al. 2007), the platform greatly minimizes manual sample handling, analysis/testing time with minimal cell perturbation, which are critical features for point-of-care applications. Clinical applications were demonstrated with assessment of neutrophil function called NETosis and metastatic potential of cancer cell.

5.1.1 Formation of neutrophil extracellular traps (NETosis)

NETosis is a novel mechanism for neutrophils to resolve an inflammation by releasing neutrophil extracellular traps (NETs), comprising of decondensed chromatin, histones and granule proteins to trap and kill pathogens (Brinkmann et al. 2004; Papayannopoulos 2017; Yipp and Kubes 2013). NETosis can be induced by a variety of

stimulus such as phorbol 12-myristate 13-acetate (PMA), calcium ionophores and certain bacteria (Konig and Andrade 2016; Parker et al. 2012). During the course of NETosis, neutrophils will undergo various morphological changes namely; 1. Cell and nucleus enlargement 2. Nucleus expansion filling cell interior 3. Nucleus envelop disintegration and releasing of NETs to cytoplasm 4. Cell membrane rupture followed by NETs releasing (Papayannopoulos 2017; Takei et al. 1996) (Figure 5.1). Previous literatures performed electrical characterization of NETosis using impedance spectroscopy (Anna et al. 2015; Schröter et al. 2013; Urban et al. 2015) and electrorotation (Griffith and Cooper 1998). However, they measured impedance spectra of bulk neutrophil population seeded on interdigitated electrodes thereby measuring average response of neutrophils rather than a single cell response. Furthermore, they utilized laborious sample preparation processes including Ficoll density gradient centrifugation and RBC lysis for neutrophil purification.

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Figure 5.1 Neutrophil Extracellular Traps (NETs) formation (Papayannopoulos 2017)

5.1.2 Cancer cell phenotyping

Cancer is one of the most lethal diseases and the second leading cause of death globally according to World Health Organization (WHO). Cancer related mortality are often associated with metastasis whereby circulating tumor cells (CTCs) start spreading to colonize other vital organs (Guan 2015). Characterization and phenotyping of CTCs are of essence in understanding disease progression as well as inventing novel therapeutic approaches. For example, in cancer management, CTCs level in blood is related to survival rate of patients and prediction of treatment outcomes (Cristofanilli et al. 2004; Krebs et al. 2010; Yan et al. 2017). Various microfluidic platforms with single-cell impedance measurement were proposed for cancer cell identification and discrimination (Chen et al. 2011; Han et al. 2007; Spencer et al. 2014b; Zhao et al. 2014). Most of the literatures showed cancer cell classification from cultured cell-lines, only Morgan et al. demonstrated spiked cancer cell (MCF-7) counting from lysed blood (Morgan and Spencer 2014). However, they required off-chip sample preparation process (lysis) which complicated device operation. Hence, there is a critical need for the development of rapid and label-free integrated platform for cancer cell sorting and phenotyping towards cancer cell bedside monitoring.

5.2 Experimental section

5.2.1 Device operation

Figure 5.2 shows the workflow of the integrated platform. Prior to sample perfusion into the device, the device was primed with 1% BSA in PBS for at least 1 h before experiments. 10 μ m polystyrene beads (Polysciences) were added into the sample as an internal reference. The sample and sheath were pumped into outlet inlet and inner inlet respectively. To focalize the sample stream, the flow rate ratio between sample and

sheath was fixed at 1:10. Prior cell collection, image capturing or impedance detection, the device was allowed to run for 1 min to reach stabilized state.

5.3 Results and discussion

5.3.1 Flow rate characterization with different sized particles

To determine the optimal flow rate condition for leukocytes, flow rate optimization of the integrated platform was first performed. 10 μ m, 7 μ m and 5 μ m beads were used to represent monocytes/neutrophils, lymphocytes and RBCs respectively. Figure 5.3 shows the flow profile of 10 μ m fluorescent beads at different stages whereas Figure 5.4 shows the flow profile of 5 and 7 μ m fluorescent beads at the sorting stage. At sorting stage, 10 μ m beads experienced inertial force ($a_p/h > 0.07$ where a_p is particle size, and h is height of the channel) and were focused near the inner wall continuing to next the stage (Figure 5.3). For smaller beads ($a_p/h < 0.07$), they recirculated to the outer wall due to dominant dean force. Thus, the smaller particles can be eliminated to the third branched channel. For the 1st stage of FRR, we observed that flow rate conditions from 140-170 μ L/min successfully focused 10 μ m to the center channel which leads to

Figure 5.2 Workflow of integrated platform for leukocyte subset sorting and impedance phenotyping

2nd stage of FRR. For the 2nd stage of FRR, the flow rate conditions ranging from 140-170 $\mu\text{L}/\text{min}$ also suffice for focusing 10 μm beads to the impedance detection region. As a result, the platform was able to sort and focus 10 μm beads to the impedance interrogation region while eliminating smaller beads to the waste.

To evaluate the sorting efficiency, the developed platform was first tested with a mixture of different sized-beads (5,7 and 10 μm) at sample flow rates of 150-170 $\mu\text{L}/\text{min}$. The high-speed microscopic images were taken at different sections of the device to show particle focusing stream (Figure 5.5A). Also, the eluent was collected from impedance outlet for downstream flow cytometry analysis. Based on flow cytometric analysis from Figure 5.5B, ~98.9% sorting purity of 10 μm beads can be achieved at 160 $\mu\text{L}/\text{min}$. We sought to evaluate additional important parameters of sorting namely enrichment ratio and concentration ratio. Enrichment ratio is a ratio of

Figure 5.3 Flow rate characterization of 10 μm beads at different device stages: (A) Composite fluorescence images and (B) intensity profiles of particle sorting, 1st FRR and 2nd FRR prior impedance detection at different sample flow rates ranging from 130 - 170 $\mu\text{L}/\text{min}$. Scale bar = 100 μm

target cell portion in the sample and initial target cell portion in the sample and concentration ratio is a ratio of sorted target cells and initial target cells. Enrichment ratio and concentration ratio were ~3 and ~2 fold respectively (Figure 5.5B).

5.3.2 Characterization of neutrophil sorting from lysed blood and diluted blood

To determine the optimal flow condition for neutrophil sorting from lysed blood, blood sample was lysed and washed with PBS. As the RBCs were lysed, the washed sample mainly comprising of leukocytes (~60% neutrophils, ~30% lymphocytes and ~10% monocytes) and platelets was perfused into the integrated platform at different flow rates and eluent from the impedance outlet was collected for flow cytometry analysis. As shown on Figure 5.6A, high speed microscopic images, the neutrophils equilibrated near the inner wall and were sorted to 2nd branch channel continuing to the

Figure 5.4 Flow rate characterization of 5 and 7 μm beads at the sorting stage: Composite fluorescence images of particle sorting at different sample flow rates ranging from 130 - 170 $\mu\text{L}/\text{min}$. Scale bar = 100 μm

next stages (FRR1 and FRR2) whereas the smaller cells such as lymphocytes, RBC debris and platelets were eliminated through 3rd branch channel. For the FRR1 and FRR2, majority of sorted neutrophils were focused to the center and proceeded to the impedance detection region. At an optimal sample flow rate of 160 μ L/min, the

Figure 5.5 Performance evaluation of the integrated platform with bead mixture (A) High-speed images for different sized bead mixture (5, 7, 10 μ m). Scale bar = 100 μ m (B) Flow cytometric analysis of sorting efficiency (left), enrichment (center) and concentration ratio (right)

integrated platform can achieve 84.66% neutrophil purity, 92.5% platelet depletion (Figure 5.6B, C). The enrichment ratio and concentration ratio were 2.86-fold and 2.07-fold respectively (Figure 5.6D, E).

Next, the neutrophil sorting from lysed blood was investigated. Blood sample was diluted by a factor of 500 and then pumped into the integrated platform at different flow rates and eluent was collected from the impedance outlet for flow cytometry analysis. Figure 5.7A shows the stacked high-speed microscopic images of the integrated platform at different flow rates. Due to high density of RBCs presence in the sample, thick RBC bands were visibly located near the outer wall (Figure 5.7A). At 170 μ L/min, RBCs completed the dean cycle (at $\sim$ 160 μ L/min) and started to recirculate to the inner wall of the channel. Also, some RBCs continued to the next stage deteriorating sorting purity as observable in high speed microscopic images (Figure 5.7A) and flow cytometric analysis of RBC depletion and platelet depletion (Figure 5.7C, D). At an optimal sample flow rate of 160 μ L/min, the integrated platform can achieve 77% neutrophil purity, 99.5% RBC depletion and 97.9% platelet elimination (Figure 5.7B, C, D). The enrichment ratio and concentration ratio were 144.64-fold and 1.12-fold respectively.

Figure 5.6 Performance evaluation of neutrophil sorting from lysed blood (A) High-speed images showing cells focusing at different flow rates and different parts of the device. Scale bar = 100 μm (B) Flow cytometric analysis of neutrophil sorting efficiency, (C) platelet depletion, (D) enrichment and (E) concentration ratio

Figure 5.7 Performance evaluation of neutrophil sorting from diluted whole blood (500 $\times$) (A) High-speed images showing cells focusing at different flow rates and different parts of the device. Scale bar = 100 μm (B) Flow cytometric analysis of neutrophil sorting efficiency, (C) RBC depletion, (D) platelet depletion, (E) neutrophil enrichment and (F) concentration ratio

5.3.3 Characterization of monocyte sorting from PBMCs

Besides neutrophil sorting, our developed platform can also be used for monocyte sorting from PBMC sample obtained from Ficoll density gradient centrifugation. PBMCs mainly consisting of lymphocytes, monocytes and platelets were pumped to the integrated platform at different flow rates and eluents from the impedance outlets were collected for flow cytometry analysis. At the sorting stage, monocyte population also equilibrated near the inner wall since monocytes and neutrophils are similar in size (Figure 5.8A). For smaller cells such as lymphocytes and platelets were eliminated to the waste through 3rd branch channel. Figure 5.8B shows >80% monocyte sorting purity can be achieved at flow rate conditions from 150-170 μ L/min. However, the device operating at 160 μ L/min had slightly higher platelet depletion (~95.6%) compared to other conditions (Figure 5.8C). The enrichment ratio and concentration ratio were ~11-fold and ~1.75-fold respectively (Figure 5.8D, E).

Figure 5.8 Performance evaluation of monocyte sorting from PBMCs (A) High-speed images showing cells focusing at different flow rates and different parts of the device. Scale bar = 100 μm (B) Flow cytometric analysis of monocyte sorting efficiency, (C) platelet depletion, (D) enrichment and (E) concentration ratio

5.3.4 Impedance identification of sorted neutrophils and monocytes

Next, we performed impedance identification of neutrophils and monocytes from different kind of samples namely lysed blood, diluted blood and PBMCs. For every samples, 10 μ m fluorescence beads were added to the samples at 100k beads/mL. Since the beads have consistent dielectric properties, they will serve as a reference for comparisons across different experiments and devices. To demonstrate impedance profile of cells, collected data were plotted as a 2D scatter distribution plot based on cell size (μ m) and opacity ($|Z_{HF}|/|Z_{LF}|$, ratio of impedance magnitude at 1.72MHz to 0.3MHz) which corresponds to cell membrane property (Holmes et al. 2009; van Berkel et al. 2011). Figure 5.9A shows impedance profile of sorted neutrophils from lysed blood spiked with 10 μ m beads. From the impedance profile, there were 4 apparent populations corresponding to 10 μ m beads and neutrophils. According to an impedance simulation, beads have higher opacity than cells due to dielectric dispersion at high frequency. In this case, the two populations with opacity around ~ 1 were corresponding to 10 μ m beads (highlighted in red), whereas the rest populations with opacity around ~ 0.85 were corresponding to neutrophils (highlighted in green). The reason that there are two apparent populations in the impedance profile for each cell type is because when the particles flow inside microchannel, they can equilibrate to two different vertical positions owing to the inertial focusing effect (Figure 5.9A (right)). The particles close to the electrodes or far from the electrodes experienced electric field strength differently thereby affecting their measured impedance responses (De Ninno et al. 2017; Spencer et al. 2016; Spencer and Morgan 2011). In Figure 5.9B, C, there was no significant difference in impedance profiles of sorted neutrophils from lysed blood and diluted blood. In term of number of events, lysed samples and diluted whole blood can provide ~ 8000 , ~ 400 neutrophils per minute respectively. Next, monocytes sorted from PBMC

Figure 5.9 Impedance profiling of different leukocytes from different blood samples The impedance profiles were represented as a 2D scatter distribution plot based on cell size ($|Z_{LF}|$, impedance magnitude at 0.3MHz) and opacity ($|Z_{HF}|/|Z_{LF}|$, ratio of impedance magnitude at 1.72MHz to 0.3MHz) (A) impedance profile of sorted neutrophils highlighted in green and spiked 10 μm beads highlighted in red (left) and illustration of particle positions inside detection channel (right), representative impedance profiles of (B) sorted neutrophils from lysed blood, (C) sorted neutrophils from diluted blood (500 $\times$) and (D) sorted monocytes from PBMCs. For every plot, the cell opacity was normalized by the average opacity of reference beads.

sample were also electrically characterized as shown in Figure 5.9D and they had a lower opacity than neutrophils due to subtle size difference which affects the membrane capacitance (proportional to the surface area). Taken together, the results show the versatility of integrated platform in leukocyte subset sorting and identification from various kinds of blood sample.

5.3.5 NETosis electrical profiling for functional assessment

To determine if the integrated platform can be used for rapid functional phenotyping, we characterized neutrophils undergoing NETosis induced by calcium ionophore (CaI). Neutrophils were stained with Hoechst for nucleus identification and SYTOX green, a cell-impermeant DNA dye. Time-lapse fluorescent imaging was then

performed to observe morphological changes during NETosis progression for 120 minutes. As shown in Figure 5.10, neutrophil size (red dotted line) and its nucleus became larger with time. The leakage of nuclear content, due to membrane permeability changes during NETosis, was visualized by SYTOX green dye after 60 min. We next evaluated the NETosis process using a plate reader and observed a significant increase in fluorescence signal over time. Glucose-treated neutrophils (30 mM) also exhibited a higher increase in fluorescence intensity as compared to untreated (control) neutrophils, which indicated an upregulation of NETosis (Figure 5.10B). We further quantified NETosis process using our developed impedance cytometer and showed that the opacity and size distribution was higher for neutrophils undergoing NETosis (NET 30 min, 60min and 120 min) as compared to neutrophils without stimulation (control, 0 min) obtained from the same individual (Figure 5.10C). The mean opacity and cell size based on the centroid of the impedance profiles from different donors ($n = 5$) were quantified and normalized with their respective control (control 0 min) to obtain relative changes. Both relative opacity and cell size were higher for NETosis neutrophils as compared to unstimulated neutrophils (Figure 5.10D). These dielectric changes-increase in opacity (increase in impedance at high frequency) and cell size (increase in impedance at low frequency) for neutrophils undergoing NETosis were similar with previous findings by Schröter et al. in which they observed elevated impedance spectra(Schröter et al. 2016). Interestingly, while a larger cell size typically corresponds to a larger surface area and decreased opacity ($\text{Opacity} \propto C_{\text{mem}}^{-1}$, $C_{\text{mem}} \propto \text{surface area}$), we observed an increase in opacity for neutrophils undergoing NETosis which could be attributed to changes in cell membrane or cytoplasm conductivity as CaI induces a massive calcium influx(Kenny et al. 2017). Griffith et al. have also reported that changes in electrorotation spectra of activated neutrophils was due to the expulsion of ions which led to an increase in cell

size and reduction of cytoplasm conductivity(Griffith and Cooper 1998). Additionally, these dielectric changes could also be associated with nucleus degradation which can be modelled as a structural transformation from a double shelled particle to a single shelled particle. Further studies will be warranted to test these hypotheses.

As a proof-of-concept for diabetes testing, we investigated the influence of glucose treatment on NETosis electrical profiles. Similar trends were observed in the impedance profile of glucose-treated neutrophils undergoing NETosis (Figure 5.10D). For both cell size and opacity, there were negligible differences between untreated and glucose-treated neutrophils at the early stage of NETosis (30min). However, the difference in cell size (glucose > untreated) became significant ($P < 0.05$) after 60 min and at 120 min. We also observed a slight increase in opacity (untreated > glucose) after 120 min, which could be associated with the larger number neutrophils undergoing NETosis(Tay et al. 2018; Wong et al. 2015). These impedance results were also consistent with our SYTOX green fluorescence spectrometry which showed higher fluorescence intensities for glucose-treated neutrophils after 60 min (Figure 5.10B).

Previous literatures have reported electrorotation(Griffith and Cooper 1998) and impedance spectroscopy(Schröter et al. 2016) for NETosis characterization and quantification. However, electrorotation suffers from low throughput (<50 cells/s), and impedance spectroscopy measures the bulk or averaged impedance spectra of neutrophil population undergoing NETosis on interdigitated electrodes. This is a major shortcoming since it is difficult to pinpoint whether the changes in electrical signal are caused by the released NETs or the structural cellular changes of neutrophils.

Figure 5.10 NETosis profiling using impedance cytometry. (A) Overlaid fluorescent (Hoechst and SYTOX Green) and brightfield images showing NETosis progression (using CaI) over the course of 2 hour. Red dotted lines highlight cell boundary determined from brightfield images. Scalebar = 10 μ m (B) Fluorescent intensity of SYTOX green for healthy neutrophils and glucose-treated neutrophils undergoing NETosis. Data are presented as mean $\pm$ s.e.m (n = 4). *P $\leq$ 0.05. (C) Representative impedance profiling of neutrophils sorted from lysed blood undergoing NETosis at different time points. Dotted lines indicate means of each cluster. (D) Average relative neutrophil cell size (left) and opacity (right) with respect to unstimulated neutrophil (0 min) for control and glucose-treated data

Additionally, laborious and time-consuming sample preparation processes (Dextran-Ficoll-Lysis, >60 min) were employed for neutrophil isolation (Schröter et al. 2016). Our developed platform addressed aforementioned issues by integrating high throughput neutrophil purification and impedance-based NETosis at single-cell resolution within minutes (<5 min).

5.3.6 Cancer cell sorting and discrimination

We expanded the concept of integrated sorting and electrical phenotyping platform to cancer cell sorting and discrimination. An integrated platform with higher height (150 μm) based on the abovementioned integrated platform was designed. Figure 5.11 shows flow rate distribution in microfluidic networks of the new design. First, the focusing behaviour of 15 μm fluorescent beads at different flow conditions was studied

Figure 5.11 Hydraulic circuit of the integrated platform for cancer cell applications: Hydraulic resistances of the microfluidic network are designed such that the flow rate is greatly reduced by 49.6 $\times$ prior impedance detection channel.

and it was found that device operating at high flow rates (190-200 $\mu\text{L}/\text{min}$) was able to deliver a majority of 15 μm beads to the impedance detection region (Figure 5.12). Next, two different breast cancer cells with different metastatic potentials namely MCF-7 and MDA-MB 231 were used to characterize the system as shown in Figure 5.13.

Figure 5.12 Flow rate characterization of 15 μm beads at different device stages: Composite fluorescence images of particle sorting at different sample flow rates ranging from 150 - 200 $\mu\text{L}/\text{min}$. Scale bar = 100 μm

Interestingly, focusing quality varies between two cell types especially for FRR1 in which a large number of MDA-MB 231 were directed to the top channel (waste) as compared to MCF-7. This may be due to a disparity in shape and size heterogeneity of both cell types. For FRR2, a large number of cells were sorted to the bottom channel (waste) for both cell types. Lastly, impedance profiling of MCF-7 and MDA-MB 231 were performed as shown in Figure 5.14. The results showed that MCF-7 were larger in size as compared to MDA-MB 231 while MDA-MB 231 was more heterogeneous in

Figure 5.13 High-speed images of cells focusing at different flow rates and different parts of the device (A) MDA-MB 231 (B) MCF-7

Figure 5.14 Characterization of cancer cells Impedance profiles of MCF-7 (left) and MDA-MB 231 (right) size and shape as evident in wide opacity distribution. This provides the possibility to discriminate different cancer cells with different metastatic potential using impedance characteristics. Overall, our data clearly demonstrated the feasibility of using the integrated platform for assessment of metastatic potential of cancer cells. The further optimizations are required for maximizing cell collection performance and handling of minimally processed blood as well as heterogeneity of cancer cell phenotypes.

5.4 Conclusion

In this chapter, a novel integrated platform for cell sorting and electrical phenotyping using impedance cytometry was proposed. The “sample in-answer out” platform offers great versatility in blood processing and it also significantly minimizes sample handling and user operation. Critically, since both cell sorting and impedance measurement are label-free, the “untouched” sorted cells after impedance measurements can be continuously collected off-chip for downstream assays. Besides leukocyte sorting, identification and counting, the developed platform was used for functional characterization of NETosis in which we observed significant differences in dielectric properties (opacity and size) between healthy and glucose treated neutrophils, as well as cancer cells with different metastatic potentials. We envision our developed platform can be further developed and translated into a point-of-care testing technology for rapid

and multi-parametric impedance-based functional immunophenotyping to identify novel biomarkers for disease diagnostics and risk stratification.

CHAPTER 6 CONCLUSIONS AND FUTURE WORKS

6.1 Conclusions

With the advent of microtechnology, the utilization of microfluidics for cell sorting and single cell studies offers various advantages such as low cost, low sample consumption, single cell scale manipulation and possibilities of integration. Despite numerous research efforts made in the past decade, most microfluidic approaches focus on optimizing a single functionality rather than developing integrated platforms for “sample in-answer out” applications.

In this dissertation, the use of impedance profiling for leukocyte activation, and the realization of an integrated platform for leukocyte sorting and impedance characterization were reported. Firstly, the combinatorial microfluidic strategy of leukocyte sorting prior impedance measurement greatly increases the detection selectivity. This was demonstrated for various leukocyte related applications namely monocyte differentiation, monocyte subtype characterization, and monocyte activation in diabetes testing. Remarkably, it was found that leukocyte impedance characteristics were associated with cardiovascular risk factors including lipid levels, C-reactive protein (CRP) and vascular functions (LnRHI) ($P < 0.05$) in T2DM patients. Secondly, both cell sorting module and impedance detection module were combined on a single biochip. The integrated platform was compatible with commonly used blood samples and greatly minimized sample handling and analysis time with minimal cell perturbation, which are attractive features for point-of-care testing applications. As proof of concept for leukocyte testing, the developed platform was used for characterization of a key defense mechanism of neutrophils called NETosis. Moreover, by changing the dimension of microfluidic network, the feasibility of a single step

cancer cell sorting and electrical phenotyping for assessment of metastatic potential was demonstrated. Overall, we believed that impedance cytometry could be a powerful tool for providing new information and insights regarding single cell biophysical and functional phenotypes for biomedical research and point-of-care applications.

6.2 Future works

There are still several improvements which can be done to enhance the capability of the proposed platform towards clinical needs and point-of-care applications.

6.2.1 Novel electrode fabrication scheme: self-aligned contact electrodes using liquid metal

Our approach to enable single cell impedance measurement was based on a conventional microfabrication where a thin metal layer is deposited to create coplanar electrodes on glass substrate. As mentioned in Chapter 4, this configuration is inferior to parallel configuration in term of detection sensitivity and detection limit. However, it is non trivial to fabricate an impedance cytometer with parallel electrode configuration since it typically requires multiple precise alignment steps. Moreover, having top and bottom parallel electrode configuration restricts the device height to the detection channel height thus limiting on-chip integration with other modules that require a different height (such as DFF). Glass etching for defining a different height is technically possible but expensive. Alternatively, sidewall electrode configurations are notably equivalent to top-bottom parallel configuration namely electroplating electrodes (Frankowski et al. 2015), conducting PDMS composite electrodes (Lewpiriyawong et al. 2010; Puttaswamy et al. 2015; Yuan et al. 2016) and 3D electrodes by Guiducci et al. (Kilchenmann et al. 2013; Rollo et al. 2017). The electroplating technique, used for creating electrodes on a channel wall, suffers from a variation in electrode structures

(especially for height) leading poorer performance than top-bottom configuration (Frankowski et al. 2015). By mixing PDMS with conductive powders such as Ag (Lewpiriyawong et al. 2010; Yuan et al. 2016) or carbon (Puttaswamy et al. 2015), one can render conductive PDMS composite which can be perfused into microchannel. Once cured, the PDMS composite will serve as a sidewall electrode. However, conducting PDMS composites may not be conductive enough for sensing applications or may require large excitation signal to enable an electrical measurement. For 3D electrodes proposed by Guiducci et al, their device required a tedious fabrication involving DRIE etching, sputtering and dry etching (Kilchenmann et al. 2013; Rollo et al. 2017). Gel patterning technique also offers an additional way of sidewall electrode fabrication. However, conductivity of metal ($>10^7$ S/m) is far more superior than that of gel (Agarose 0.4 S/m (Kandadai et al. 2012)). Also, the stability of gel material over time is questionable. To solve aforementioned issues, it is aimed to develop self-aligned contact electrodes using gallium based liquid metals. Due to their excellent properties, gallium and its alloy have gained considerable attention for various microfluidics applications including coulter counter (Khoshmanesh et al. 2017; Richards et al. 2012). Although, there are coulter counter demonstrations using liquid metal previously, the device was not optimized for leukocyte phenotyping applications as the detection region was excessively larger than the cells. Also, the device did not employ differential measurement scheme which is generally known to increase sensitivity of the detection. Figure 6.1A shows our proposed design which is a microchannel with step lateral electrode loading channel. When liquid metal is injected into the lateral channel, it will not flow into the high hydraulic resistance detection channel while exposing to the detection channel. The preliminary studies were performed where Eutectic Gallium–Indium (EGaIn) was loaded into electrode channels. When FITC solution was injected

at 10 μ L/min, we observed that FITC solution flowed into the electrode channels since those channels can provide low hydraulic resistance path (Figure 6.1B). Future plans include using other kinds of liquid metal for patterning and design optimization to achieve desire electrical performances.

6.2.2 Reduction of equilibrium positions in the integrated inertial-impedance cytometry platform

As mentioned in Chapter 5, particles traversing inside the integrated platform experience strong inertial focusing and equilibrate across the longer channel width (top and bottom) resulting in two apparent clusters in an impedance profile for each particle type. This adversely affects signal analysis (e.g. centroid determination) as impedance profile cannot be quantified directly and additional step is required to extract clusters. Although there were several works (Caselli et al. 2018; Caselli et al. 2017; De Ninno et al. 2017; Spencer et al. 2016) demonstrated the use of multiple electrodes configuration to sense particle's position along the channel height, it is still required post-processing data analysis. Moreover, the implementation of multiple electrodes causes the overall larger detection region which may compromise system throughput as well as sensitivity.

Figure 6.1 (A) Schematic of microfluidic device with self-aligned liquid metal electrode. Green box provides close-up images of detection region. (B) Preliminary studies using EGaIn

Alternatively, active particle focusing using DEP has been widely used in certain impedance cytometers (Haandbaek et al. 2014; Mernier et al. 2012) but device operation become more complicated and cautions must be taken as active particle focusing might affect impedance readout. Therefore, it is imperative to find a mean to inherently reduce particle equilibrium positions from two positions to one position. Not only this would greatly simplify the post processing, it can be exploited to enhance signal sensitivity.

6.2.3 On integrated functionality: post active sorting

As shown in Chapter 4, it was demonstrated that certain leukocyte subtypes, notably low-density neutrophils can be identify using impedance cytometry. It would be more insightful if impedance-based sorting of target cells can be deployed to acquire target cells for other aspects of cell studies such as cell culture or other conventional assays. Although, several sorting system based on impedance signatures were reported for 3D printing (Schoendube et al. 2015) and sperm sorting (de Wagenaar et al. 2016), the throughput still remains as a major challenge. For those aforementioned works, their reported throughput were only 9 events/min and 5 events/sec respectively. We envision that by increasing throughput, impedance-based sorting system could potentially be a label-free alternative to conventional FACs/MACs systems.

6.2.4 Potential applications of impedance cytometry

Immunology research and cancer research

The proposed strategy and integrated platform can be extended to immunology research and cancer research. For immunology research, the platform can be optimized for the biophysical or functional studies of other leukocytes such lymphocytes. As shown in chapter 4 and chapter 5, impedance cytometry can be used to discriminate cancer cells from normal cells based on cell size and opacity. Although the specificity

and sensitivity of this technique is not as superior as affinity-based techniques such as conventional FACs and MACs, it can still be used to explore the associations between single cell biophysical properties and cancer cell types. Further studies are required to evaluate its efficiency for cancer cells discrimination. To add specificity for impedance-based detection, several literatures demonstrated the possibility of using antibody conjugated beads (Holmes and Morgan 2010) or graphene nanoplates (Han and Han 2015) to tag specific markers on cells resulting in enhanced electrical detection. However, using antibody tagging renders the technique as non label-free.

Bioreactor monitoring and cell line analysis

In these applications, impedance cytometry as an electrical characterization tool can be used in several aspects namely 1. identification of phenotypes 2. cell status monitoring. For the first point, it could be used to explore a link between physical properties and industrially relevant phenotypes of cell lines such as growth, product quality, productivity and stability. Secondly, the technique may possibly use to assess status of cells whether they are live, dead or activated. By coupling with any separation technique, it is possible to provide selection of live cells or effective producers to maximize yield of a bioreactor. Noteworthy, as a label-free technique, it can operate in rapid and continuous manner with minimal effects on cell viability and cell activation.

REFERENCES

1. Abeles, R.D., McPhail, M.J., Sowter, D., Antoniadou, C.G., Vergis, N., Vijay, G.K., Xystrakis, E., Khamri, W., Shawcross, D.L., Ma, Y., Wendon, J.A., Vergani, D., 2012. CD14, CD16 and HLA-DR reliably identifies human monocytes and their subsets in the context of pathologically reduced HLA-DR expression by CD14(hi) /CD16(neg) monocytes: Expansion of CD14(hi) /CD16(pos) and contraction of CD14(lo) /CD16(pos) monocytes in acute liver failure. *Cytometry. Part A : the journal of the International Society for Analytical Cytology* 81(10), 823-834.
2. Altschuler, S.J., Wu, L.F., Cellular Heterogeneity: Do Differences Make a Difference? *Cell* 141(4), 559-563.
3. Amini, H., Lee, W., Di Carlo, D., 2014. Inertial microfluidic physics. *Lab on a Chip* 14(15), 2739-2761.
4. Anna, S., Johannes, W., Andreas, N., Chokri, C., Angela, R.-W., Gerald, G., 2015. Miniaturized wound sensors based on detection of extracellular chromatin. *Lecture Notes on Impedance Spectroscopy*, pp. 15-20. CRC Press.
5. Asami, K., 2002. Characterization of biological cells by dielectric spectroscopy. *Journal of Non-Crystalline Solids* 305(1-3), 268-277.
6. Asami, K., Hanai, T., 1992. Dielectric monitoring of biological cell sedimentation. *Colloid and Polymer Science* 270(1), 78-84.
7. Asmolov, E.S., 1999. The inertial lift on a spherical particle in a plane Poiseuille flow at large channel Reynolds number. *Journal of Fluid Mechanics* 381, 63-87.
8. Barat, D., Spencer, D., Benazzi, G., Mowlem, M.C., Morgan, H., 2012. Simultaneous high speed optical and impedance analysis of single particles with a microfluidic cytometer. *Lab on a Chip* 12(1), 118-126.
9. Becker, F.F., Wang, X.B., Huang, Y., Pethig, R., Vykoukal, J., Gascoyne, P.R., 1995. Separation of human breast cancer cells from blood by differential dielectric affinity. *Proceedings of the National Academy of Sciences of the United States of America* 92(3), 860-864.
10. Bernabini, C., Holmes, D., Morgan, H., 2011. Micro-impedance cytometry for detection and analysis of micron-sized particles and bacteria. *Lab on a Chip* 11(3), 407-412.
11. Beyrau, M., Bodkin, J.V., Nourshargh, S., 2012. Neutrophil heterogeneity in health and disease: a revitalized avenue in inflammation and immunity. *Open Biology* 2(11).
12. Bhagat, A.A.S., Kuntaegowdanahalli, S.S., Papautsky, I., 2008a. Continuous particle separation in spiral microchannels using dean flows and differential migration. *Lab on a Chip* 8(11), 1906-1914.
13. Bhagat, A.A.S., Kuntaegowdanahalli, S.S., Papautsky, I., 2008b. Continuous particle separation in spiral microchannels using dean flows and differential migration. *Lab on a Chip* 8(11), 1906.
14. Boden, G., Rao, A.K., 2006. Platelet and monocyte activation by hyperglycemia and hyperinsulinemia in healthy subjects AU - Vaidyula, Vijender R. *Platelets* 17(8), 577-585.
15. Bordi, F., Cametti, C., De Luca, F., Gili, T., Misasi, R., Sorice, M., Circella, A., Garofalo, T., 2001. Structural alteration of erythrocyte membrane during storage: a combined electrical conductometric and flow-cytometric study. *Zeitschrift für Naturforschung C* 56(9-10), 857-864.
16. Bordi, F., Cametti, C., Rosi, A., Calcabrini, A., 1993. Frequency domain electrical conductivity measurements of the passive electrical properties of human lymphocytes. *Biochimica et Biophysica Acta (BBA) - Biomembranes* 1153(1), 77-88.

17. Bose, S., Singh, R., Hanewich-Hollatz, M., Shen, C., Lee, C.-H., Dorfman, D.M., Karp, J.M., Karnik, R., 2013. Affinity flow fractionation of cells via transient interactions with asymmetric molecular patterns. *Sci. Rep.* 3.
18. Bosshart, H., Heinzelmann, M., 2016. THP-1 cells as a model for human monocytes. *Annals of translational medicine* 4(21), 438-438.
19. Boyette, L.B., Macedo, C., Hadi, K., Elinoff, B.D., Walters, J.T., Ramaswami, B., Chalasani, G., Taboas, J.M., Lakkis, F.G., Metes, D.M., 2017. Phenotype, function, and differentiation potential of human monocyte subsets. *PLOS ONE* 12(4), e0176460.
20. Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D.S., Weinrauch, Y., Zychlinsky, A., 2004. Neutrophil Extracellular Traps Kill Bacteria. *Science* 303(5663), 1532-1535.
21. Brody, J.P., Osborn, T.D., Forster, F.K., Yager, P., 1995. A Planar Microfabricated Fluid Filter. *Solid-State Sensors and Actuators, 1995 and Eurosensors IX.. Transducers '95. The 8th International Conference on*, pp. 779-782.
22. Caduff, A., Livshits, L., Hayashi, Y., Feldman, Y., 2004. Cell membrane response on D-glucose studied by dielectric spectroscopy. Erythrocyte and ghost suspensions. *The Journal of Physical Chemistry B* 108(36), 13827-13830.
23. Carmona-Rivera, C., Kaplan, M.J., 2013. Low density granulocytes: a distinct class of neutrophils in systemic autoimmunity. *Seminars in immunopathology* 35(4), 455-463.
24. Caselli, F., De Ninno, A., Reale, R., Businaro, L., Bisegna, P., 2018. A novel wiring scheme for standard chips enabling high-accuracy impedance cytometry. *Sensors and Actuators B: Chemical* 256, 580-589.
25. Caselli, F., Reale, R., Nodargi, N., Bisegna, P., 2017. Numerical Investigation of a Novel Wiring Scheme Enabling Simple and Accurate Impedance Cytometry. *Micromachines* 8(9), 283.
26. Che, J., Yu, V., Garon, E.B., Goldman, J.W., Di Carlo, D., 2017. Biophysical isolation and identification of circulating tumor cells. *Lab on a Chip* 17(8), 1452-1461.
27. Chen, J., Xue, C., Zhao, Y., Chen, D., Wu, M.-H., Wang, J., 2015. Microfluidic Impedance Flow Cytometry Enabling High-Throughput Single-Cell Electrical Property Characterization. *International Journal of Molecular Sciences* 16(5), 9804.
28. Chen, J., Zheng, Y., Tan, Q., Shojaei-Baghini, E., Zhang, Y.L., Li, J., Prasad, P., You, L., Wu, X.Y., Sun, Y., 2011. Classification of cell types using a microfluidic device for mechanical and electrical measurement on single cells. *Lab on a Chip* 11(18), 3174-3181.
29. Chen, X., Cui, D.-F., 2009. Microfluidic devices for sample pretreatment and applications. *Microsyst Technol* 15(5), 667-676.
30. Cheung, K., Gawad, S., Renaud, P., 2005. Impedance spectroscopy flow cytometry: On-chip label-free cell differentiation. *Cytometry Part A* 65A(2), 124-132.
31. Cheung, K.C., Di Berardino, M., Schade-Kampmann, G., Hebeisen, M., Pierzchalski, A., Bocsi, J., Mittag, A., Tárnok, A., 2010. Microfluidic impedance-based flow cytometry. *Cytometry Part A* 77(7), 648-666.
32. Coulter, W.H., 1956. High speed automatic blood cell counter and cell size analyzer. *Proc Natl Electron Conf*, pp. 1034-1040.
33. Cristofanilli, M., Budd, G.T., Ellis, M.J., Stopeck, A., Matera, J., Miller, M.C., Reuben, J.M., Doyle, G.V., Allard, W.J., Terstappen, L.W.M.M., Hayes, D.F., 2004. Circulating Tumor Cells, Disease

Progression, and Survival in Metastatic Breast Cancer. *New England Journal of Medicine* 351(8), 781-791.

34. Cros, J., Cagnard, N., Woollard, K., Patey, N., Zhang, S.Y., Senechal, B., Puel, A., Biswas, S.K., Moshous, D., Picard, C., Jais, J.P., D'Cruz, D., Casanova, J.L., Trouillet, C., Geissmann, F., 2010. Human CD14^{dim} monocytes patrol and sense nucleic acids and viruses via TLR7 and TLR8 receptors. *Immunity* 33(3), 375-386.

35. Cruz, J.M., García-Diego, F.J., 1998. Dielectrophoretic motion of oblate spheroidal particles. Measurements of motion of red blood cells using the Stokes method. *Journal of Physics D: Applied Physics* 31(14), 1745.

36. Daigneault, M., Preston, J.A., Marriott, H.M., Whyte, M.K.B., Dockrell, D.H., 2010. The identification of markers of macrophage differentiation in PMA-stimulated THP-1 cells and monocyte-derived macrophages. *PloS one* 5(1), e8668-e8668.

37. De Ninno, A., Errico, V., Bertani, F.R., Businaro, L., Bisegna, P., Caselli, F., 2017. Coplanar electrode microfluidic chip enabling accurate sheathless impedance cytometry. *Lab on a Chip*.

38. de Wagenaar, B., Dekker, S., de Boer, H.L., Bomer, J.G., Olthuis, W., van den Berg, A., Segerink, L.I., 2016. Towards microfluidic sperm refinement: impedance-based analysis and sorting of sperm cells. *Lab on a Chip* 16(8), 1514-1522.

39. Delamaire, M., Maugendre, D., Moreno, M., Le Goff, M.C., Allannic, H., Genetet, B., 1997. Impaired leucocyte functions in diabetic patients. *Diabetic Medicine* 14(1), 29-34.

40. Demierre, N., Braschler, T., Linderholm, P., Seger, U., van Lintel, H., Renaud, P., 2007. Characterization and optimization of liquid electrodes for lateral dielectrophoresis. *Lab on a Chip* 7(3), 355-365.

41. Deng, T., Prentiss, M., Whitesides, G.M., 2002. Fabrication of magnetic microfiltration systems using soft lithography. *Applied Physics Letters* 80(3), 461-463.

42. Deng, Y., Ye, J., Luo, Q., Huang, Z., Peng, Y., Xiong, G., Guo, Y., Jiang, H., Li, J., 2016. Low-Density Granulocytes Are Elevated in Mycobacterial Infection and Associated with the Severity of Tuberculosis. *PLOS ONE* 11(4), e0153567.

43. Dhar, M., Wong, J., Che, J., Matsumoto, M., Grogan, T., Elashoff, D., Garon, E.B., Goldman, J.W., Sollier Christen, E., Di Carlo, D., Kulkarni, R.P., 2018. Evaluation of PD-L1 expression on vortex-isolated circulating tumor cells in metastatic lung cancer. *Scientific Reports* 8(1), 2592.

44. Di Biasio, A., Cametti, C., 2010. D-glucose-induced alterations in the electrical parameters of human erythrocyte cell membrane. *Bioelectrochemistry* 77(2), 151-157.

45. Di Carlo, D., 2009. Inertial microfluidics. *Lab on a Chip* 9(21), 3038-3046.

46. Di Carlo, D., Irimia, D., Tompkins, R.G., Toner, M., 2007. Continuous inertial focusing, ordering, and separation of particles in microchannels. *Proceedings of the National Academy of Sciences* 104(48), 18892-18897.

47. Donath, E., Egger, M., Pastushenko, V.P., 1990. Dielectric behavior of the anion-exchange protein of human red blood cells: theoretical analysis and comparison to electrorotation data. *Bioelectrochemistry and Bioenergetics* 23(3), 337-360.

48. Du, E., Ha, S., Diez-Silva, M., Dao, M., Suresh, S., Chandrakasan, A.P., 2013. Electric impedance microflow cytometry for characterization of cell disease states. *Lab on a Chip* 13(19), 3903-3909.

49. Emmelkamp, J., Wolbers, F., Andersson, H., Dacosta, R.S., Wilson, B.C., Vermes, I., van den Berg, A., 2004. The potential of autofluorescence for the detection of single living cells for label-free cell sorting in microfluidic systems. *Electrophoresis* 25(21-22), 3740-3745.
50. Ermolina, I., Poleyeva, Y., Feldman, Y., Ginzburg, B.Z., Schlesinger, M., 2001. Study of normal and malignant white blood cells by time domain dielectric spectroscopy. *IEEE Transactions on Dielectrics and Electrical Insulation* 8(2), 253-261.
51. Escate, R., Padro, T., Badimon, L., 2016. LDL accelerates monocyte to macrophage differentiation: Effects on adhesion and anoikis. *Atherosclerosis* 246, 177-186.
52. Evander, M., Ricco, A.J., Morser, J., Kovacs, G.T.A., Leung, L.L.K., Giovangrandi, L., 2013. Microfluidic impedance cytometer for platelet analysis. *Lab on a Chip* 13(4), 722-729.
53. Faivre, M., Abkarian, M., Bickraj, K., Stone, H.A., 2006. Geometrical focusing of cells in a microfluidic device: an approach to separate blood plasma. *Biorheology* 43(2), 147-160.
54. Fernandez-Ruiz, I., Puchalska, P., Narasimhulu, C.A., Sengupta, B., Parthasarathy, S., 2016. Differential lipid metabolism in monocytes and macrophages: influence of cholesterol loading. *Journal of Lipid Research* 57(4), 574-586.
55. Frankowski, M., Simon, P., Bock, N., El-Hasni, A., Schnakenberg, U., Neukammer, J., 2015. Simultaneous optical and impedance analysis of single cells: A comparison of two microfluidic sensors with sheath flow focusing. *Engineering in Life Sciences* 15(3), 286-296.
56. Fricke, H., 1924. A mathematical treatment of the electric conductivity and capacity of disperse systems I. The electric conductivity of a suspension of homogeneous spheroids. *Physical Review* 24(5), 575.
57. Fricke, H., 1925a. The Electric Capacity of Suspensions of Red Corpuscles of a Dog. *Physical Review* 26(5), 682-687.
58. Fricke, H., 1925b. The electric capacity of suspensions with special reference to blood. *The Journal of general physiology* 9(2), 137-152.
59. Fricke, H., 1925c. A mathematical treatment of the electric conductivity and capacity of disperse systems II. The capacity of a suspension of conducting spheroids surrounded by a non-conducting membrane for a current of low frequency. *Physical Review* 26(5), 678.
60. Fricke, H., Curtis, H.J., 1935. Electric impedance of suspensions of leucocytes. *Nature* 135, 436.
61. Fricke, H., Morse, S., 1925. The electric resistance and capacity of blood for frequencies between 800 and 4½ million cycles. *The Journal of general physiology* 9(2), 153.
62. Fu, A.Y., Spence, C., Scherer, A., Arnold, F.H., Quake, S.R., 1999. A microfabricated fluorescence-activated cell sorter. *Nature biotechnology* 17(11), 1109-1111.
63. Fu, J., Tobin, M.C., Thomas, L.L., 2014. Neutrophil-like low-density granulocytes are elevated in patients with moderate to severe persistent asthma. *Annals of Allergy, Asthma & Immunology* 113(6), 635-640.e632.
64. Fukuda, S., Schmid-Schönbein, G.W., 2002. Centrifugation attenuates the fluid shear response of circulating leukocytes. *Journal of Leukocyte Biology* 72(1), 133-139.
65. Gabriel, C., Gabriel, S., Corthout, E., 1996. The dielectric properties of biological tissues: I. Literature survey. *Physics in Medicine and Biology* 41(11), 2231.
66. Gascoyne, P., Mahidol, C., Ruchirawat, M., Satayavivad, J., Watcharasit, P., Becker, F.F., 2002. Microsample preparation by dielectrophoresis: isolation of malaria. *Lab on a Chip* 2(2), 70-75.

67. Gascoyne, P., Pethig, R., Satayavivad, J., Becker, F.F., Ruchirawat, M., 1997. Dielectrophoretic detection of changes in erythrocyte membranes following malarial infection. *Biochimica et Biophysica Acta (BBA)-Biomembranes* 1323(2), 240-252.
68. Gascoyne, P.R.C., Shim, S., Noshari, J., Becker, F.F., Stemke-Hale, K., 2013. Correlations between the Dielectric Properties and Exterior Morphology of Cells Revealed by Dielectrophoretic Field-Flow Fractionation. *Electrophoresis* 34(7), 1042-1050.
69. Gawad, S., Cheung, K., Seger, U., Bertsch, A., Renaud, P., 2004. Dielectric spectroscopy in a micromachined flow cytometer: theoretical and practical considerations. *Lab Chip* 4(3), 241-251.
70. Gawad, S., Schild, L., Renaud, P., 2001. Micromachined impedance spectroscopy flow cytometer for cell analysis and particle sizing. *Lab on a Chip* 1(1), 76-82.
71. Georgieva, R., Neu, B., Shilov, V., Knippel, E., Budde, A., Latza, R., Donath, E., Kieseewetter, H., Bäuml, H., 1998. Low frequency electrorotation of fixed red blood cells. *Biophysical journal* 74(4), 2114-2120.
72. Giulietti, A., van Etten, E., Overbergh, L., Stoffels, K., Bouillon, R., Mathieu, C., 2007. Monocytes from type 2 diabetic patients have a pro-inflammatory profile. 1,25-Dihydroxyvitamin D(3) works as anti-inflammatory. *Diabetes research and clinical practice* 77(1), 47-57.
73. Godino, N., Vereshchagina, E., Gorkin, R., Ducrée, J., 2014. Centrifugal automation of a triglyceride bioassay on a low-cost hybrid paper-polymer device. *Microfluidics and Nanofluidics* 16(5), 895-905.
74. Gordon, S., Taylor, P.R., 2005. Monocyte and macrophage heterogeneity. *Nat Rev Immunol* 5(12), 953-964.
75. Griffith, A.W., Cooper, J.M., 1998. Single-cell measurements of human neutrophil activation using electrorotation. *Analytical chemistry* 70(13), 2607-2612.
76. Guan, X., 2015. Cancer metastases: challenges and opportunities. *Acta Pharmaceutica Sinica B* 5(5), 402-418.
77. Haandbaek, N., With, O., Burgel, S.C., Heer, F., Hierlemann, A., 2014. Resonance-enhanced microfluidic impedance cytometer for detection of single bacteria. *Lab on a Chip* 14(17), 3313-3324.
78. Han, A., Yang, L., Frazier, A.B., 2007. Quantification of the Heterogeneity in Breast Cancer Cell Lines Using Whole-Cell Impedance Spectroscopy. *Clinical Cancer Research* 13(1), 139-143.
79. Han, K.-H., Bruno Frazier, A., 2004. Continuous magnetophoretic separation of blood cells in microdevice format. *Journal of Applied Physics* 96(10), 5797-5802.
80. Han, K.-H., Frazier, A.B., 2006. Paramagnetic capture mode magnetophoretic microseparator for high efficiency blood cell separations. *Lab on a Chip* 6(2), 265-273.
81. Han, S.-I., Han, K.-H., 2015. Electrical Detection Method for Circulating Tumor Cells Using Graphene Nanoplates. *Analytical Chemistry* 87(20), 10585-10592.
82. Hanai, T., Asami, K., Koizumi, N., 1979. Dielectric theory of concentrated suspensions of shell. *Bull. Inst. Chem. Res. Kyoto Univ.* 57.
83. Hartge, M.M., Unger, T., Kintscher, U., 2007. The endothelium and vascular inflammation in diabetes. *Diabetes and Vascular Disease Research* 4(2), 84-88.
84. Hassan, U., Ghonge, T., Reddy Jr, B., Patel, M., Rappleye, M., Taneja, I., Tanna, A., Healey, R., Manusry, N., Price, Z., Jensen, T., Berger, J., Hasnain, A., Flaugh, E., Liu, S., Davis, B., Kumar, J.,

- White, K., Bashir, R., 2017. A point-of-care microfluidic biochip for quantification of CD64 expression from whole blood for sepsis stratification 8, 15949.
85. Hassan, U., Reddy, B., Damhorst, G., Sonoiki, O., Ghonge, T., Yang, C., Bashir, R., 2015. A microfluidic biochip for complete blood cell counts at the point-of-care. *Technology* 3(4), 201-213.
 86. Hassan, U., Watkins, N.N., Reddy Jr, B., Damhorst, G., Bashir, R., 2016. Microfluidic differential immunocapture biochip for specific leukocyte counting. *Nat. Protocols* 11(4), 714-726.
 87. Hayashi, Y., Katsumoto, Y., Omori, S., Yasuda, A., Asami, K., Kaibara, M., Uchimura, I., 2010. Dielectric Coagulometry: A New Approach To Estimate Venous Thrombosis Risk. *Analytical Chemistry* 82(23), 9769-9774.
 88. Hayashi, Y., Livshits, L., Caduff, A., Feldman, Y., 2003. Dielectric spectroscopy study of specific glucose influence on human erythrocyte membranes. *Journal of Physics D: Applied Physics* 36(4), 369.
 89. Hayashi, Y., Oshige, I., Katsumoto, Y., Omori, S., Yasuda, A., Asami, K., 2007. Temporal variation of dielectric properties of preserved blood. *Physics in medicine and biology* 53(1), 295.
 90. Henderson, B., Kinahan, D.J., Ducrée, J., 2016. The Centrifugal Microfluidic: Lab-on-a-Disc Platform. In: Dixit, C.K., Kaushik, A. (Eds.), *Microfluidics for Biologists: Fundamentals and Applications*, pp. 115-144. Springer International Publishing, Cham.
 91. Henson, R., 2016. Flow Cytometry Data Reader and Visualization (<http://www.mathworks.com/matlabcentral/fileexchange/8430-flow-cytometry-data-reader-and-visualization>). MATLAB Central File Exchange.
 92. Herteman, N., Vargas, A., Lavoie, J.-P., 2017. Characterization of Circulating Low-Density Neutrophils Intrinsic Properties in Healthy and Asthmatic Horses. *Scientific Reports* 7(1), 7743.
 93. Höber, R., 1910. Eine Methode, die elektrische Leitfähigkeit im Innern von Zellen zu messen. *Pflüger's Archiv für die gesamte Physiologie des Menschen und der Tiere* 133(4-6), 237-253.
 94. Höber, R., 1912. Ein zweites Verfahren, die Leitfähigkeit im Innern von Zellen zu messen. *Pflüger's Archiv für die gesamte Physiologie des Menschen und der Tiere* 148(4-5), 189-221.
 95. Hoffman, R., Johnson, T., Britt, W., 1981. Flow cytometric electronic direct current volume and radiofrequency impedance measurements of single cells and particles. *Cytometry Part A* 1(6), 377-384.
 96. Holmes, D., Morgan, H., 2010. Single Cell Impedance Cytometry for Identification and Counting of CD4 T-Cells in Human Blood Using Impedance Labels. *Analytical Chemistry* 82(4), 1455-1461.
 97. Holmes, D., Pettigrew, D., Reccius, C.H., Gwyer, J.D., van Berkel, C., Holloway, J., Davies, D.E., Morgan, H., 2009. Leukocyte analysis and differentiation using high speed microfluidic single cell impedance cytometry. *Lab on a Chip* 9(20), 2881-2889.
 98. Holmes, D., She, J.K., Roach, P.L., Morgan, H., 2007. Bead-based immunoassays using a microchip flow cytometer. *Lab on a Chip* 7(8), 1048-1056.
 99. Homsy, A., van der Wal, P.D., Doll, W., Schaller, R., Korsatko, S., Ratzer, M., Ellmerer, M., Pieber, T.R., Nicol, A., de Rooij, N.F., 2012. Development and validation of a low cost blood filtration element separating plasma from undiluted whole blood. *Biomicrofluidics* 6(1), 12804-128049.
 100. Hong, J., Yoon, D.S., Kim, S.K., Kim, T.S., Kim, S., Pak, E.Y., No, K., 2005. AC frequency characteristics of coplanar impedance sensors as design parameters. *Lab Chip* 5(3), 270-279.

101. Hou, H.W., Bhagat, A.A.S., Lee, W.C., Huang, S., Han, J., Lim, C.T., 2011. Microfluidic Devices for Blood Fractionation. *Micromachines* 2(3), 319.
102. Hou, H.W., Petchakup, C., Tay, H.M., Tam, Z.Y., Dalan, R., Chew, D.E.K., Li, K.H.H., Boehm, B.O., 2016. Rapid and label-free microfluidic neutrophil purification and phenotyping in diabetes mellitus. *Scientific Reports* 6, 29410.
103. Hou, H.W., Warkiani, M.E., Khoo, B.L., Li, Z.R., Soo, R.A., Tan, D.S.-W., Lim, W.-T., Han, J., Bhagat, A.A.S., Lim, C.T., 2013. Isolation and retrieval of circulating tumor cells using centrifugal forces. *Scientific reports* 3.
104. Hu, X., Arnold, W.M., Zimmermann, U., 1990. Alterations in the electrical properties of T and B lymphocyte membranes induced by mitogenic stimulation. Activation monitored by electro-rotation of single cells. *Biochimica et biophysica acta* 1021(2), 191-200.
105. Huang, L.R., Cox, E.C., Austin, R.H., Sturm, J.C., 2004. Continuous Particle Separation Through Deterministic Lateral Displacement. *Science* 304(5673), 987-990.
106. Huang, R., Barber, T.A., Schmidt, M.A., Tompkins, R.G., Toner, M., Bianchi, D.W., Kapur, R., Flejter, W.L., 2008. A microfluidics approach for the isolation of nucleated red blood cells (NRBCs) from the peripheral blood of pregnant women. *Prenatal diagnosis* 28(10), 892-899.
107. Huang, S.-B., Zhao, Y., Chen, D., Liu, S.-L., Luo, Y., Chiu, T.-K., Wang, J., Chen, J., Wu, M.-H., 2015. Classification of Cells with Membrane Staining and/or Fixation Based on Cellular Specific Membrane Capacitance and Cytoplasm Conductivity. *Micromachines* 6(2), 163.
108. Hur, S.C., Mach, A.J., Carlo, D.D., 2011. High-throughput size-based rare cell enrichment using microscale vortices. *Biomicrofluidics* 5(2), 022206.
109. Hywel, M., Tao, S., David, H., Shady, G., Nicolas, G.G., 2007. Single cell dielectric spectroscopy. *Journal of Physics D: Applied Physics* 40(1), 61.
110. Iliescu, C., Barbarini, E., Avram, M., Xu, G., Avram, A., 2008. Microfluidic device for continuous magnetophoretic separation of red blood cells. *Design, Test, Integration and Packaging of MEMS/MOEMS, 2008. MEMS/MOEMS 2008. Symposium on*, pp. 279-281.
111. Inglis, D.W., Riehn, R., Austin, R.H., Sturm, J.C., 2004. Continuous microfluidic immunomagnetic cell separation. *Applied Physics Letters* 85(21), 5093-5095.
112. Inglis, D.W., Riehn, R., Sturm, J.C., Austin, R.H., 2006. Microfluidic high gradient magnetic cell separation. *Journal of Applied Physics* 99(8), -.
113. Irimajiri, A., Asami, K., Ichinowatari, T., Kinoshita, Y., 1987a. Passive electrical properties of the membrane and cytoplasm of cultured rat basophil leukemia cells. I. Dielectric behavior of cell suspensions in 0.01–500 MHz and its simulation with a single-shell model. *Biochimica et Biophysica Acta (BBA) - Biomembranes* 896(2), 203-213.
114. Irimajiri, A., Asami, K., Ichinowatari, T., Kinoshita, Y., 1987b. Passive electrical properties of the membrane and cytoplasm of cultured rat basophil leukemia cells. II. Effects of osmotic perturbation. *Biochimica et Biophysica Acta (BBA) - Biomembranes* 896(2), 214-223.
115. Irimajiri, A., Hanai, T., Inouye, A., 1979. A dielectric theory of "multi-stratified shell" model with its application to a lymphoma cell. *Journal of theoretical biology* 78(2), 251-269.
116. Jensen, E.C., 2012. Use of Fluorescent Probes: Their Effect on Cell Biology and Limitations. *The Anatomical Record: Advances in Integrative Anatomy and Evolutionary Biology* 295(12), 2031-2036.

117. Ji, H.M., Samper, V., Chen, Y., Heng, C.K., Lim, T.M., Yobas, L., 2008. Silicon-based microfilters for whole blood cell separation. *Biomedical microdevices* 10(2), 251-257.
118. Jones, P.V., Staton, S.J., Hayes, M.A., 2011. Blood cell capture in a sawtooth dielectrophoretic microchannel. *Analytical and bioanalytical chemistry* 401(7), 2103-2111.
119. Kandadai, M.A., Raymond, J.L., Shaw, G.J., 2012. Comparison of electrical conductivities of various brain phantom gels: Developing a 'Brain Gel Model'. *Materials science & engineering. C, Materials for biological applications* 32(8), 2664-2667.
120. Kantar, A., Giorgi, P.L., Curatola, G., Fiorini, R., 1991. Alterations in membrane fluidity of diabetic polymorphonuclear leukocytes. *Biochemical Medicine and Metabolic Biology* 46(3), 422-426.
121. Kantari, C., Pederzoli-Ribeil, M., Witko-Sarsat, V., 2008. The role of neutrophils and monocytes in innate immunity. *Contributions to microbiology* 15, 118-146.
122. Karabacak, N.M., Spuhler, P.S., Fachin, F., Lim, E.J., Pai, V., Ozkumur, E., Martel, J.M., Kojic, N., Smith, K., Chen, P.-i., Yang, J., Hwang, H., Morgan, B., Trautwein, J., Barber, T.A., Stott, S.L., Maheswaran, S., Kapur, R., Haber, D.A., Toner, M., 2014. Microfluidic, marker-free isolation of circulating tumor cells from blood samples. *Nature protocols* 9, 694.
123. Kenny, E.F., Herzig, A., Kruger, R., Muth, A., Mondal, S., Thompson, P.R., Brinkmann, V., Bernuth, H.V., Zychlinsky, A., 2017. Diverse stimuli engage different neutrophil extracellular trap pathways. *eLife* 6.
124. Khoo, B.L., Warkiani, M.E., Tan, D.S., Bhagat, A.A., Irwin, D., Lau, D.P., Lim, A.S., Lim, K.H., Krisna, S.S., Lim, W.T., Yap, Y.S., Lee, S.C., Soo, R.A., Han, J., Lim, C.T., 2014. Clinical validation of an ultra high-throughput spiral microfluidics for the detection and enrichment of viable circulating tumor cells. *PLoS One* 9(7), e99409.
125. Khoshmanesh, K., Tang, S.-Y., Zhu, J.Y., Schaefer, S., Mitchell, A., Kalantar-zadeh, K., Dickey, M.D., 2017. Liquid metal enabled microfluidics. *Lab on a Chip* 17(6), 974-993.
126. Kilchenmann, S.C., Rollo, E., Bianchi, E., Guiducci, C., 2013. Metal-coated silicon micropillars for freestanding 3D-electrode arrays in microchannels. *Sensors and Actuators B: Chemical* 185, 713-719.
127. Kim, U., Soh, H.T., 2009. Simultaneous sorting of multiple bacterial targets using integrated Dielectrophoretic-Magnetic Activated Cell Sorter. *Lab on a Chip* 9(16), 2313-2318.
128. Kirkwood, J.G., 1939. The dielectric polarization of polar liquids. *The Journal of Chemical Physics* 7(10), 911-919.
129. Konig, M.F., Andrade, F., 2016. A Critical Reappraisal of Neutrophil Extracellular Traps and NETosis Mimics Based on Differential Requirements for Protein Citrullination. *Frontiers in Immunology* 7(461).
130. Krebs, M.G., Hou, J.-M., Ward, T.H., Blackhall, F.H., Dive, C., 2010. Circulating tumour cells: their utility in cancer management and predicting outcomes. *Therapeutic Advances in Medical Oncology* 2(6), 351-365.
131. Kuntaegowdanahalli, S.S., Bhagat, A.A.S., Kumar, G., Papautsky, I., 2009. Inertial microfluidics for continuous particle separation in spiral microchannels. *Lab on a Chip* 9(20), 2973-2980.
132. Küttel, C., Nascimento, E., Demierre, N., Silva, T., Braschler, T., Renaud, P., Oliva, A.G., 2007. Label-free detection of *Babesia bovis* infected red blood cells using impedance spectroscopy on a microfabricated flow cytometer. *Acta tropica* 102(1), 63-68.

133. Kwon, T., Prentice, H., Oliveira, J.D., Madziva, N., Warkiani, M.E., Hamel, J.-F.P., Han, J., 2017. Microfluidic Cell Retention Device for Perfusion of Mammalian Suspension Culture. *Scientific Reports* 7(1), 6703.
134. Lee, M.G., Choi, S., Kim, H.-J., Lim, H.K., Kim, J.-H., Huh, N., Park, J.-K., 2011. Inertial blood plasma separation in a contraction–expansion array microchannel. *Applied Physics Letters* 98(25), 253702.
135. Lenshof, A., Ahmad-Tajudin, A., Järås, K., Swärd-Nilsson, A.-M., Åberg, L., Marko-Varga, G., Malm, J., Lilja, H., Laurell, T., 2009. Acoustic Whole Blood Plasmapheresis Chip for Prostate Specific Antigen Microarray Diagnostics. *Analytical Chemistry* 81(15), 6030-6037.
136. Lewpiriyawong, N., Yang, C., Lam, Y.C., 2010. Continuous sorting and separation of microparticles by size using AC dielectrophoresis in a PDMS microfluidic device with 3-D conducting PDMS composite electrodes. *Electrophoresis* 31(15), 2622-2631.
137. Li, X., Chen, W., Liu, G., Lu, W., Fu, J., 2014. Continuous-flow microfluidic blood cell sorting for unprocessed whole blood using surface-micromachined microfiltration membranes. *Lab on a Chip* 14(14), 2565-2575.
138. Linden, M.D., Jackson, D.E., 2010. Platelets: Pleiotropic roles in atherogenesis and atherothrombosis. *The International Journal of Biochemistry & Cell Biology* 42(11), 1762-1766.
139. Linderholm, P., Renaud, P., 2005. Comment on "AC frequency characteristics of coplanar impedance sensors as design parameters" by Jongin Hong, Dae Sung Yoon, Sung Kwan Kim, Tae Song Kim, Sanghyo Kim, Eugene Y. Pak and Kwangsoo No, *Lab Chip*, 2005, 5, 270. *Lab Chip* 5(12), 1416-1417; author reply 1418.
140. Livshits, L., Caduff, A., Talary, M., Feldman, Y., 2006. Dielectric response of biconcave erythrocyte membranes to d-and l-glucose. *Journal of Physics D: Applied Physics* 40(1), 15.
141. Livshits, L., Caduff, A., Talary, M.S., Lutz, H.U., Hayashi, Y., Puzenko, A., Shendrik, A., Feldman, Y., 2009. The role of GLUT1 in the sugar-induced dielectric response of human erythrocytes. *The Journal of Physical Chemistry B* 113(7), 2212-2220.
142. Mach, A.J., Carlo, D.D., 2010. Continuous scalable blood filtration device using inertial microfluidics. *Biotechnology and Bioengineering* 107(2), 302-311.
143. Mach, A.J., Kim, J.H., Arshi, A., Hur, S.C., Di Carlo, D., 2011. Automated cellular sample preparation using a Centrifuge-on-a-Chip. *Lab on a Chip* 11(17), 2827-2834.
144. Marchalot, J., Fouillet, Y., Achard, J.-L., 2014. Multi-step microfluidic system for blood plasma separation: architecture and separation efficiency. *Microfluidics and Nanofluidics* 17(1), 167-180.
145. Maxwell, J.C., 1881. *A treatise on electricity and magnetism*. Clarendon press.
146. Mernier, G., Duqi, E., Renaud, P., 2012. Characterization of a novel impedance cytometer design and its integration with lateral focusing by dielectrophoresis. *Lab on a Chip* 12(21), 4344-4349.
147. Michelson, A.D., Barnard, M.R., Krueger, L.A., Valeri, C.R., Furman, M.I., 2001. Circulating Monocyte-Platelet Aggregates Are a More Sensitive Marker of In Vivo Platelet Activation Than Platelet Surface P-Selectin. *Studies in Baboons, Human Coronary Intervention, and Human Acute Myocardial Infarction* 104(13), 1533-1537.
148. Miller, B., Jimenez, M., Bridle, H., 2016a. Cascading and Parallelising Curvilinear Inertial Focusing Systems for High Volume, Wide Size Distribution, Separation and Concentration of Particles 6, 36386.

149. Miller, B., Jimenez, M., Bridle, H., 2016b. Cascading and Parallelising Curvilinear Inertial Focusing Systems for High Volume, Wide Size Distribution, Separation and Concentration of Particles. *Sci Rep-Uk* 6.
150. Min, D., Brooks, B., Wong, J., Salomon, R., Bao, W., Harrisberg, B., Twigg, S.M., Yue, D.K., McLennan, S.V., 2012. Alterations in monocyte CD16 in association with diabetes complications. *Mediators of inflammation* 2012, 649083.
151. Mirza, S., Hossain, M., Mathews, C., Martinez, P., Pino, P., Gay, J.L., Rentfro, A., McCormick, J.B., Fisher-Hoch, S.P., 2012. Type 2-diabetes is associated with elevated levels of TNF-alpha, IL-6 and adiponectin and low levels of leptin in a population of Mexican Americans: a cross-sectional study. *Cytokine* 57(1), 136-142.
152. Mizuno, M., Yamada, M., Mitamura, R., Ike, K., Toyama, K., Seki, M., 2013. Magnetophoresis-Integrated Hydrodynamic Filtration System for Size- and Surface Marker-Based Two-Dimensional Cell Sorting. *Analytical Chemistry* 85(16), 7666-7673.
153. Morgan, H., Holmes, D., Green, N.G., 2006. High speed simultaneous single particle impedance and fluorescence analysis on a chip. *Current Applied Physics* 6(3), 367-370.
154. Morgan, H., Spencer, D., 2014. Microfluidic impedance cytometry for blood cell analysis. *Microfluidics Med. Appl* 36, 213.
155. Nagrath, S., Sequist, L.V., Maheswaran, S., Bell, D.W., Irimia, D., Ulkus, L., Smith, M.R., Kwak, E.L., Digumarthy, S., Muzikansky, A., Ryan, P., Balis, U.J., Tompkins, R.G., Haber, D.A., Toner, M., 2007. Isolation of rare circulating tumour cells in cancer patients by microchip technology. *Nature* 450(7173), 1235-1239.
156. Nakashima, Y., Hata, S., Yasuda, T., 2010. Blood plasma separation and extraction from a minute amount of blood using dielectrophoretic and capillary forces. *Sensors and Actuators B: Chemical* 145(1), 561-569.
157. Nam, J., Lim, H., Kim, D., Shin, S., 2011. Separation of platelets from whole blood using standing surface acoustic waves in a microchannel. *Lab on a Chip* 11(19), 3361-3364.
158. Nicolás-Ávila, J.Á., Adrover, J.M., Hidalgo, A., 2017. Neutrophils in Homeostasis, Immunity, and Cancer. *Immunity* 46(1), 15-28.
159. Nivedita, N., Garg, N., Lee, A.P., Papautsky, I., 2017. A high throughput microfluidic platform for size-selective enrichment of cell populations in tissue and blood samples. *The Analyst* 142(14), 2558-2569.
160. Ookawara, S., Street, D., Ogawa, K., 2006. Numerical study on development of particle concentration profiles in a curved microchannel. *Chemical engineering science* 61(11), 3714-3724.
161. Papayannopoulos, V., 2017. Neutrophil extracellular traps in immunity and disease. *Nature Reviews Immunology* 18, 134.
162. Park, J.-S., Jung, H.-I., 2009. Multiorifice Flow Fractionation: Continuous Size-Based Separation of Microspheres Using a Series of Contraction/Expansion Microchannels. *Analytical Chemistry* 81(20), 8280-8288.
163. Parker, H., Dragunow, M., Hampton, M.B., Kettle, A.J., Winterbourn, C.C., 2012. Requirements for NADPH oxidase and myeloperoxidase in neutrophil extracellular trap formation differ depending on the stimulus. *Journal of Leukocyte Biology* 92(4), 841-849.
164. Patkó, Z., Császár, A., Acsády, G., Öry, I., Takács, É., Fűrész, J., 2012. Elevation of monocyte-platelet aggregates is an early marker of type 2 diabetes. *Interventional Medicine & Applied Science* 4(4), 181-185.

165. Petchakup, C., Li, K., Hou, H., 2017. Advances in Single Cell Impedance Cytometry for Biomedical Applications. *Micromachines* 8(3), 87.
166. Petchakup, C., Tay, H.M., Yeap, W.H., Dalan, R., Wong, S.C., Li, K.H.H., Hou, H.W., 2018. Label-free leukocyte sorting and impedance-based profiling for diabetes testing. *Biosensors and Bioelectronics* 118, 195-203.
167. Petersson, F., Aberg, L., Sward-Nilsson, A.M., Laurell, T., 2007. Free flow acoustophoresis: microfluidic-based mode of particle and cell separation. *Anal Chem* 79(14), 5117-5123.
168. Petersson, F., Nilsson, A., Holm, C., Jonsson, H., Laurell, T., 2004. Separation of lipids from blood utilizing ultrasonic standing waves in microfluidic channels. *The Analyst* 129(10), 938-943.
169. Petersson, F., Nilsson, A., Jönsson, H., Laurell, T., 2005. Carrier Medium Exchange through Ultrasonic Particle Switching in Microfluidic Channels. *Analytical Chemistry* 77(5), 1216-1221.
170. Pethig, R., 2017. Where Is Dielectrophoresis (DEP) Going? *Journal of The Electrochemical Society* 164(5), B3049-B3055.
171. Pethig, R., Bressler, V., Carswell-Crumpton, C., Chen, Y., Foster-Haje, L., García-Ojeda, M.E., Lee, R.S., Lock, G.M., Talar, M.S., Tate, K.M., 2002. Dielectrophoretic studies of the activation of human T lymphocytes using a newly developed cell profiling system. *Electrophoresis* 23(13), 2057-2063.
172. Pethig, R., Talar, M.S., 2007. Dielectrophoretic detection of membrane morphology changes in Jurkat T-cells undergoing etoposide-induced apoptosis. *IET Nanobiotechnol* 1(1), 2-9.
173. Piacentini, N., Mernier, G., Tornay, R., Renaud, P., 2011. Separation of platelets from other blood cells in continuous-flow by dielectrophoresis field-flow-fractionation. *Biomicrofluidics* 5(3), 034122.
174. Polevaya, Y., Ermolina, I., Schlesinger, M., Ginzburg, B.Z., Feldman, Y., 1999. Time domain dielectric spectroscopy study of human cells. II. Normal and malignant white blood cells. *Biochimica et biophysica acta* 1419(2), 257-271.
175. Pommer, M.S., Zhang, Y., Keerthi, N., Chen, D., Thomson, J.A., Meinhart, C.D., Soh, H.T., 2008. Dielectrophoretic separation of platelets from diluted whole blood in microfluidic channels. *Electrophoresis* 29(6), 1213-1218.
176. Progatzy, F., Dallman, M.J., Lo Celso, C., 2013. From seeing to believing: labelling strategies for in vivo cell-tracking experiments. *Interface Focus* 3(3), 20130001.
177. Puttaswamy, S.V., Sivashankar, S., Chen, R.-J., Chin, C.-K., Chang, H.-Y., Liu, C.H., 2010. Enhanced cell viability and cell adhesion using low conductivity medium for negative dielectrophoretic cell patterning. *Biotechnology Journal* 5(10), 1005-1015.
178. Puttaswamy, S.V., Xue, P., Kang, Y., Ai, Y., 2015. Simple and low cost integration of highly conductive three-dimensional electrodes in microfluidic devices. *Biomedical microdevices* 17(1), 4.
179. Qin, Z., 2012. The use of THP-1 cells as a model for mimicking the function and regulation of monocytes and macrophages in the vasculature. *Atherosclerosis* 221(1), 2-11.
180. Rafeie, M., Zhang, J., Asadnia, M., Li, W., Warkiani, M.E., 2016. Multiplexing slanted spiral microchannels for ultra-fast blood plasma separation. *Lab on a Chip* 16(15), 2791-2802.
181. Ramachandraiah, H., Svahn, H.A., Russom, A., 2017. Inertial microfluidics combined with selective cell lysis for high throughput separation of nucleated cells from whole blood. *RSC Advances* 7(47), 29505-29514.

182. Richards, A.L., Dickey, M.D., Kennedy, A.S., Buckner, G.D., 2012. Design and demonstration of a novel micro-Coulter counter utilizing liquid metal electrodes. *Journal of Micromechanics and Microengineering* 22(11), 115012.
183. Robinson, M., Marks, H., Hinsdale, T., Maitland, K., Cote, G., 2017. Rapid isolation of blood plasma using a cascaded inertial microfluidic device. *Biomicrofluidics* 11(2), 024109.
184. Rodriguez-Trujillo, R., Castillo-Fernandez, O., Garrido, M., Arundell, M., Valencia, A., Gomila, G., 2008. High-speed particle detection in a micro-Coulter counter with two-dimensional adjustable aperture. *Biosensors and Bioelectronics* 24(2), 290-296.
185. Rodriguez-Trujillo, R., Mills, C., Samitier, J., Gomila, G., 2007a. Low cost micro-Coulter counter with hydrodynamic focusing. *Microfluidics and Nanofluidics* 3(2), 171-176.
186. Rodriguez-Trujillo, R., Mills, C.A., Samitier, J., Gomila, G., 2007b. Low cost micro-Coulter counter with hydrodynamic focusing. *Microfluidics and Nanofluidics* 3(2), 171-176.
187. Rollo, E., Tenaglia, E., Genolet, R., Bianchi, E., Harari, A., Coukos, G., Guiducci, C., 2017. Label-free identification of activated T lymphocytes through tridimensional microsensors on chip. *Biosensors and Bioelectronics* 94, 193-199.
188. Rubinshtein, R., Kuvin, J.T., Soffler, M., Lennon, R.J., Lavi, S., Nelson, R.E., Pumper, G.M., Lerman, L.O., Lerman, A., 2010. Assessment of endothelial function by non-invasive peripheral arterial tonometry predicts late cardiovascular adverse events. *European Heart Journal* 31(9), 1142-1148.
189. Rüegg, C., 2006. Leukocytes, inflammation, and angiogenesis in cancer: fatal attractions. *Journal of Leukocyte Biology* 80(4), 682-684.
190. Ryu, H., Choi, K., Qu, Y., Kwon, T., Lee, J.S., Han, J., 2017. Patient-Derived Airway Secretion Dissociation Technique To Isolate and Concentrate Immune Cells Using Closed-Loop Inertial Microfluidics. *Analytical Chemistry* 89(10), 5549-5556.
191. Schoendube, J., Wright, D., Zengerle, R., Koltay, P., 2015. Single-cell printing based on impedance detection. *Biomicrofluidics* 9(1), 014117.
192. Schröter, A., Rösen-Wolff, A., Gerlach, G., 2013. Impedance-based infection model of human neutrophils. *Lecture Notes on Impedance Spectroscopy* 4, 59.
193. Schröter, A., Wegner, S., Bulst, M., Parker, H., Rösen-Wolff, A., Gerlach, G., 2016. Impedance model of immune reaction leading to NETosis and comparison with in vitro measurements. *Sensors and Actuators B: Chemical* 236, 947-953.
194. Schwan, H., 1968a. Electrode polarization impedance and measurements in biological material. *Annals of the New York Academy of Sciences* 148(1), 191-209.
195. Schwan, H., 1968b. ELECTRODE POLARIZATION IMPEDANCE AND MEASUREMENTS IN BIOLOGICAL MATERIALS*. *Annals of the New York Academy of Sciences* 148(1), 191-209.
196. Schwan, H., 1994. Electrical properties of tissues and cell suspensions: mechanisms and models. *Engineering in Medicine and Biology Society, 1994. Engineering Advances: New Opportunities for Biomedical Engineers. Proceedings of the 16th Annual International Conference of the IEEE*, pp. A70-A71 vol. 71. IEEE.
197. Schwan, H.P., 1956. Electrical properties of tissue and cell suspensions. *Advances in biological and medical physics* 5, 147-209.
198. Schwan, H.P., 1963. Determination of biological impedances. *Physical techniques in biological research* 6(Part B), 323-407.

199. Scott, R., Sethu, P., Harnett, C., 2008. Three-dimensional hydrodynamic focusing in a microfluidic Coulter counter. *Review of Scientific Instruments* 79(4), 046104.
200. Segr, G., Silberberg, A., 1961. Radial Particle Displacements in Poiseuille Flow of Suspensions. *Nature* 189, 209.
201. Sethu, P., Sin, A., Toner, M., 2006. Microfluidic diffusive filter for apheresis (leukapheresis). *Lab on a Chip* 6(1), 83-89.
202. Shaker, M., Colella, L., Caselli, F., Bisegna, P., Renaud, P., 2014. An impedance-based flow microcytometer for single cell morphology discrimination. *Lab Chip* 14(14), 2548-2555.
203. Shanmugam, N., Reddy, M.A., Guha, M., Natarajan, R., 2003. High Glucose-Induced Expression of Proinflammatory Cytokine and Chemokine Genes in Monocytic Cells. *Diabetes* 52(5), 1256.
204. Shantsila, E., Wrigley, B., Tapp, L., Apostolakis, S., Montoro-Garcia, S., Drayson, M.T., Lip, G.Y.H., 2011. Immunophenotypic characterization of human monocyte subsets: possible implications for cardiovascular disease pathophysiology. *Journal of Thrombosis and Haemostasis* 9(5), 1056-1066.
205. Sheng, Y., Jun, Z., Chao, P., Dan, Y., Gursel, A., Haiping, D., Yonggang, Z., Weihua, L., 2015. An integrated dielectrophoresis-active hydrophoretic microchip for continuous particle filtration and separation. *Journal of Micromechanics and Microengineering* 25(8), 084010.
206. Sheng, Y., Jun, Z., Dan, Y., Weihua, L., 2017. Hybrid microfluidics combined with active and passive approaches for continuous cell separation. *Electrophoresis* 38(2), 238-249.
207. Shi, J., Huang, H., Stratton, Z., Huang, Y., Huang, T.J., 2009. Continuous particle separation in a microfluidic channel via standing surface acoustic waves (SSAW). *Lab on a Chip* 9(23), 3354-3359.
208. Simon, P., Frankowski, M., Bock, N., Neukammer, J., 2016. Label-free whole blood cell differentiation based on multiple frequency AC impedance and light scattering analysis in a micro flow cytometer. *Lab on a Chip* 16(12), 2326-2338.
209. Sims, C.E., Allbritton, N.L., 2007. Analysis of single mammalian cells on-chip. *Lab on a Chip* 7(4), 423-440.
210. Sollier, E., Cubizolles, M., Fouillet, Y., Achard, J.-L., 2010. Fast and continuous plasma extraction from whole human blood based on expanding cell-free layer devices. *Biomedical microdevices* 12(3), 485-497.
211. Sollier, E., Go, D.E., Che, J., Gossett, D.R., O'Byrne, S., Weaver, W.M., Kummer, N., Rettig, M., Goldman, J., Nickols, N., McCloskey, S., Kulkarni, R.P., Di Carlo, D., 2014. Size-selective collection of circulating tumor cells using Vortex technology. *Lab on a Chip* 14(1), 63-77.
212. Spencer, D., Caselli, F., Bisegna, P., Morgan, H., 2016. High accuracy particle analysis using sheathless microfluidic impedance cytometry. *Lab on a Chip*.
213. Spencer, D., Elliott, G., Morgan, H., 2014a. A sheath-less combined optical and impedance micro-cytometer. *Lab on a Chip* 14(16), 3064-3073.
214. Spencer, D., Hollis, V., Morgan, H., 2014b. Microfluidic impedance cytometry of tumour cells in blood. *Biomicrofluidics* 8(6), 064124.
215. Spencer, D., Morgan, H., 2011. Positional dependence of particles in microfluidic impedance cytometry. *Lab on a Chip* 11(7), 1234-1239.
216. Sprangers, S., de Vries, T.J., Everts, V., 2016. Monocyte Heterogeneity: Consequences for Monocyte-Derived Immune Cells. *Journal of Immunology Research* 2016, 1475435.

217. Sun, T., Green, N., Morgan, H., 2008. Analytical and numerical modeling methods for impedance analysis of single cells on-chip. *Nano* 03(01), 55-63.
218. Sun, T., Green, N.G., Gawad, S., Morgan, H., 2007a. Analytical electric field and sensitivity analysis for two microfluidic impedance cytometer designs. *IET Nanobiotechnology* 1(5), 69-79.
219. Sun, T., Morgan, H., 2010. Single-cell microfluidic impedance cytometry: a review. *Microfluidics and Nanofluidics* 8(4), 423-443.
220. Sun, T., Morgan, H., Green, N.G., 2007b. Analytical solutions of ac electrokinetics in interdigitated electrode arrays: Electric field, dielectrophoretic and traveling-wave dielectrophoretic forces. *Physical Review E* 76(4), 046610.
221. Sun, T., van Berkel, C., Green, N.G., Morgan, H., 2009. Digital signal processing methods for impedance microfluidic cytometry. *Microfluidics and Nanofluidics* 6(2), 179-187.
222. Surowiec, A., Stuchly, S.S., Izaguirre, C., 1986. Dielectric properties of human B and T lymphocytes at frequencies from 20 kHz to 100 MHz. *Phys Med Biol* 31(1), 43-53.
223. Takagi, J., Yamada, M., Yasuda, M., Seki, M., 2005. Continuous particle separation in a microchannel having asymmetrically arranged multiple branches. *Lab Chip* 5(7), 778-784.
224. Takei, H., Araki, A., Watanabe, H., Ichinose, A., Sendo, F., 1996. Rapid killing of human neutrophils by the potent activator phorbol 12-myristate 13-acetate (PMA) accompanied by changes different from typical apoptosis or necrosis. *Journal of Leukocyte Biology* 59(2), 229-240.
225. Tao, S., Shady, G., Nicolas, G.G., Hywel, M., 2007. Dielectric spectroscopy of single cells: time domain analysis using Maxwell's mixture equation. *Journal of Physics D: Applied Physics* 40(1), 1.
226. Tay, H.M., Dalan, R., Li, K.H.H., Boehm, B.O., Hou, H.W., 2018. A Novel Microdevice for Rapid Neutrophil Purification and Phenotyping in Type 2 Diabetes Mellitus. *Small* 14(6), 1702832.
227. Toner, M., Irimia, D., 2005. Blood-on-a-chip. *Annual review of biomedical engineering* 7, 77-103.
228. Trøseid, M., Nestvold, T.K., Rudi, K., Thoresen, H., Nielsen, E.W., Lappegård, K.T., 2013. Plasma Lipopolysaccharide Is Closely Associated With Glycemic Control and Abdominal Obesity. *Diabetes Care* 36(11), 3627.
229. Urban, G., Wöllenstein, J., Kieninger, J., Schröter, A., Rösen-Wolff, A., Gerlach, G., 2015. Impedance Model of Immune Reaction Leading to NETosis. *Procedia Engineering* 120, 564-569.
230. Valero, A., Braschler, T., Renaud, P., 2010. A unified approach to dielectric single cell analysis: Impedance and dielectrophoretic force spectroscopy. *Lab on a Chip* 10(17), 2216-2225.
231. van Berkel, C., Gwyer, J.D., Deane, S., Green, N., Holloway, J., Hollis, V., Morgan, H., 2011. Integrated systems for rapid point of care (PoC) blood cell analysis. *Lab on a Chip* 11(7), 1249-1255.
232. van Oostrom, A.J., van Wijk, J.P., Sijmonsma, T.P., Rabelink, T.J., Castro Cabezas, M., 2004. Increased expression of activation markers on monocytes and neutrophils in type 2 diabetes. *The Netherlands journal of medicine* 62(9), 320-325.
233. VanDelinder, V., Groisman, A., 2006. Separation of Plasma from Whole Human Blood in a Continuous Cross-Flow in a Molded Microfluidic Device. *Analytical Chemistry* 78(11), 3765-3771.
234. Vykoukal, D.M., Gascoyne, P.R.C., Vykoukal, J., 2009. Dielectric characterization of complete mononuclear and polymorphonuclear blood cell subpopulations for label-free discrimination. *Integrative Biology* 1(7), 477-484.

235. Wang, J., Lu, W., Tang, C., Liu, Y., Sun, J., Mu, X., Zhang, L., Dai, B., Li, X., Zhuo, H., Jiang, X., 2015. Label-Free Isolation and mRNA Detection of Circulating Tumor Cells from Patients with Metastatic Lung Cancer for Disease Diagnosis and Monitoring Therapeutic Efficacy. *Analytical Chemistry* 87(23), 11893-11900.
236. Wang, S., Sarenac, D., Chen, M.H., Huang, S.H., Giguel, F.F., Kuritzkes, D.R., Demirci, U., 2012. Simple filter microchip for rapid separation of plasma and viruses from whole blood. *International journal of nanomedicine* 7, 5019-5028.
237. Wang, X., Becker, F.F., Gascoyne, P.R., 2002. Membrane dielectric changes indicate induced apoptosis in HL-60 cells more sensitively than surface phosphatidylserine expression or DNA fragmentation. *Biochimica et biophysica acta* 1564(2), 412-420.
238. Ward, D.M., 2011. Conventional apheresis therapies: a review. *Journal of clinical apheresis* 26(5), 230-238.
239. Warkiani, M.E., Khoo, B.L., Tan, D.S.-W., Bhagat, A.A.S., Lim, W.-T., Yap, Y.S., Lee, S.C., Soo, R.A., Han, J., Lim, C.T., 2014. An ultra-high-throughput spiral microfluidic biochip for the enrichment of circulating tumor cells. *The Analyst* 139(13), 3245-3255.
240. Warkiani, M.E., Tay, A.K.P., Guan, G., Han, J., 2015. Membrane-less microfiltration using inertial microfluidics 5, 11018.
241. Watarai, H., 2013. Continuous Separation Principles Using External Microaction Forces. *Annual Review of Analytical Chemistry* 6(1), 353-378.
242. Watkins, N., Venkatesan, B.M., Toner, M., Rodriguez, W., Bashir, R., 2009. A robust electrical microcytometer with 3-dimensional hydrofocusing. *Lab on a Chip* 9(22), 3177-3184.
243. Watkins, N.N., Hassan, U., Damhorst, G., Ni, H., Vaid, A., Rodriguez, W., Bashir, R., 2013. Microfluidic CD4⁺ and CD8⁺ T Lymphocyte Counters for Point-of-Care HIV Diagnostics Using Whole Blood. *Science Translational Medicine* 5(214), 214ra170-214ra170.
244. Whitesides, G.M., 2006. The origins and the future of microfluidics. *Nature* 442(7101), 368-373.
245. Wildgruber, M., Aschenbrenner, T., Wendorff, H., Czubba, M., Glinzer, A., Haller, B., Schiemann, M., Zimmermann, A., Berger, H., Eckstein, H.-H., Meier, R., Wohlgemuth, W.A., Libby, P., Zerneck, A., 2016. The “Intermediate” CD14(++)CD16(+) monocyte subset increases in severe peripheral artery disease in humans. *Scientific Reports* 6, 39483.
246. Wilding, P., Kricka, L.J., Cheng, J., Hvizia, G., Shoffner, M.A., Fortina, P., 1998. Integrated Cell Isolation and Polymerase Chain Reaction Analysis Using Silicon Microfilter Chambers. *Analytical Biochemistry* 257(2), 95-100.
247. Willis, M.R., 1980. Dielectric and electronic properties of biological materials by R Pethig. pp 376. John Wiley & Sons, Chichester and New York. 1979. £15. *Biochemical Education* 8(1), 31-31.
248. Wilson, E.H., Weninger, W., Hunter, C.A., 2010. Trafficking of immune cells in the central nervous system. *The Journal of Clinical Investigation* 120(5), 1368-1379.
249. Wolff, A., Perch-Nielsen, I.R., Larsen, U.D., Friis, P., Goranovic, G., Poulsen, C.R., Kutter, J.P., Telleman, P., 2003. Integrating advanced functionality in a microfabricated high-throughput fluorescent-activated cell sorter. *Lab Chip* 3(1), 22-27.
250. Wong, K.L., Tai, J.J.-Y., Wong, W.-C., Han, H., Sem, X., Yeap, W.-H., Kourilsky, P., Wong, S.-C., 2011. Gene expression profiling reveals the defining features of the classical, intermediate, and nonclassical human monocyte subsets. *Blood* 118(5), e16-e31.

251. Wong, S.L., Demers, M., Martinod, K., Gallant, M., Wang, Y., Goldfine, A.B., Kahn, C.R., Wagner, D.D., 2015. Diabetes primes neutrophils to undergo NETosis, which impairs wound healing. *Nature Medicine* 21, 815.
252. Wynn, T.A., Chawla, A., Pollard, J.W., 2013. Macrophage biology in development, homeostasis and disease. *Nature* 496, 445.
253. Xia, H., Strachan, B.C., Gifford, S.C., Shevkoplyas, S.S., 2016. A high-throughput microfluidic approach for 1000-fold leukocyte reduction of platelet-rich plasma. *Scientific Reports* 6, 35943.
254. Xia, Y., Whitesides, G.M., 1998. Soft Lithography. *Angewandte Chemie International Edition* 37(5), 550-575.
255. Yager, P., Edwards, T., Fu, E., Helton, K., Nelson, K., Tam, M.R., Weigl, B.H., 2006. Microfluidic diagnostic technologies for global public health. *Nature* 442(7101), 412-418.
256. Yamada, M., Nakashima, M., Seki, M., 2004. Pinched Flow Fractionation: Continuous Size Separation of Particles Utilizing a Laminar Flow Profile in a Pinched Microchannel. *Analytical Chemistry* 76(18), 5465-5471.
257. Yan, W.-T., Cui, X., Chen, Q., Li, Y.-F., Cui, Y.-H., Wang, Y., Jiang, J., 2017. Circulating tumor cell status monitors the treatment responses in breast cancer patients: a meta-analysis. *Scientific Reports* 7, 43464.
258. Yang, J., Huang, Y., Wang, X., Wang, X.-B., Becker, F.F., Gascoyne, P.R.C., 1999. Dielectric Properties of Human Leukocyte Subpopulations Determined by Electrorotation as a Cell Separation Criterion. *Biophysical Journal* 76(6), 3307-3314.
259. Yang, S., Undar, A., Zahn, J.D., 2006. A microfluidic device for continuous, real time blood plasma separation. *Lab on a Chip* 6(7), 871-880.
260. Yeo, D.C., Wiraja, C., Zhou, Y., Tay, H.M., Xu, C., Hou, H.W., 2015. Interference-free Micro/nanoparticle Cell Engineering by Use of High-Throughput Microfluidic Separation. *ACS Applied Materials & Interfaces* 7(37), 20855-20864.
261. Yipp, B.G., Kubes, P., 2013. NETosis: how vital is it? *Blood* 122(16), 2784-2794.
262. Yuan, W., Zhensong, X., Mark, A.C., John, N., Yi, Z., Chen, W., Yu, S., 2016. Embedded silver PDMS electrodes for single cell electrical impedance spectroscopy. *Journal of Micromechanics and Microengineering* 26(9), 095006.
263. Zhang, J., Yan, S., Alici, G., Nguyen, N.-T., Di Carlo, D., Li, W., 2014. Real-time control of inertial focusing in microfluidics using dielectrophoresis (DEP). *RSC Advances* 4(107), 62076-62085.
264. Zhao, Y., Chen, D., Li, H., Luo, Y., Deng, B., Huang, S.-B., Chiu, T.-K., Wu, M.-H., Long, R., Hu, H., Wang, J., Chen, J., 2013a. A microfluidic system enabling continuous characterization of specific membrane capacitance and cytoplasm conductivity of single cells in suspension. *Biosensors and Bioelectronics* 43, 304-307.
265. Zhao, Y., Chen, D., Luo, Y., Li, H., Deng, B., Huang, S.-B., Chiu, T.-K., Wu, M.-H., Long, R., Hu, H., Zhao, X., Yue, W., Wang, J., Chen, J., 2013b. A microfluidic system for cell type classification based on cellular size-independent electrical properties. *Lab on a Chip* 13(12), 2272-2277.
266. Zhao, Y., Jiang, M., Chen, D., Zhao, X., Xue, C., Hao, R., Yue, W., Wang, J., Chen, J., 2016. Single-Cell Electrical Phenotyping Enabling the Classification of Mouse Tumor Samples. *Scientific Reports* 6, 19487.

267. Zhao, Y., Zhao, X.T., Chen, D.Y., Luo, Y.N., Jiang, M., Wei, C., Long, R., Yue, W.T., Wang, J.B., Chen, J., 2014. Tumor cell characterization and classification based on cellular specific membrane capacitance and cytoplasm conductivity. *Biosensors and Bioelectronics* 57, 245-253.
268. Zheng, S., Tai, Y.C., Kasdan, H., 2005. A micro device for separation of erythrocytes and leukocytes in human blood. *Conference proceedings : ... Annual International Conference of the IEEE Engineering in Medicine and Biology Society. IEEE Engineering in Medicine and Biology Society. Annual Conference* 1, 1024-1027.
269. Zheng, Y., Shojaei-Baghini, E., Wang, C., Sun, Y., 2013. Microfluidic characterization of specific membrane capacitance and cytoplasm conductivity of singlecells. *Biosensors and Bioelectronics* 42, 496-502.
270. Zhou, Y., Yang, D., Zhou, Y., Khoo, B.L., Han, J., Ai, Y., 2018. Characterizing Deformability and Electrical Impedance of Cancer Cells in a Microfluidic Device. *Analytical Chemistry* 90(1), 912-919.
271. Ziervogel, H., Glaser, R., Schadow, D., Heymann, S., 1986. Electrorotation of lymphocytes—The influence of membrane events and nucleus. *Bioscience reports* 6(11), 973-982.