Optofluidic chip for living cell detection and culture

Yu, Jiaqing

2014

Yu, J. (2014). Optofluidic chip for living cell detection and culture. Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/61042>

<https://doi.org/10.32657/10356/61042>

**NANYANG
TECHNOLOGICAL
UNIVERSITY**

OPTOFLUIDIC CHIP FOR LIVING CELL
DETECTION AND CULTURE

YU JIAQING

**OPTOFLUIDIC CHIP FOR LIVING CELL
DETECTION AND CULTURE**

**YU JIAQING
SCHOOL OF ELECTRICAL AND ELECTRONIC
ENGINEERING
2014**

2014

OPTOFLUIDIC CHIP FOR LIVING CELL DETECTION AND CULTURE

YU JIAQING

School of Electrical and Electronic Engineering

A thesis submitted to the Nanyang Technological University
in partial fulfillment of the requirement for the degree of
Doctor of Philosophy

2014

ACKNOWLEDGEMENTS

First and foremost, I would like to express my sincere appreciation to Professor Liu Ai Qun, who has acted as a prudent advisor and a caring mentor throughout these years. His constant dedication to education and enthusiasm towards research always inspires me to push myself further towards higher achievement. He is a caring mentor who had encouraged me not only to be patient in research achievement, but also emphasized in the practice of balance of life and health.

Next, I am grateful to Professor Kathy Luo Qian and the members from the School of Chemical and Biomedical Engineering, for her invaluable guidance and resource support in my research work on cell culture and bio-sensor development. I am also thankful to the guidance from Dr Chin Lip Ket, Dr Yang Yi and Dr Huang Wei, who has given me full support on the experiment techniques and theoretical analysis for my research. I like to thank all my current and ex-group members: Dr Zhu Weiming, Dr Yu Yefeng, Dr Lei Lei, Mr Li Zhenguo, Ms Xiong Sha, Mr Fu Yi, Mr Shahnawaz Pukkeyil Shamsuddi and Mr Song Qinghua for their tremendous help and enlightening discussions.

In addition, this work would not be possible without the assistance of the technicians in VALENS centre of excellence and Photonics Lab 1. They have provided me with technical supports for the setup of experiments and the development of fabrication processes.

Finally, I am deepest indebted to my mother and other family members for their understanding and support. Last but not least, my heartfelt appreciation is also extended to all my friends who in one way or another contributed to the pursuit of my research career.

SUMMARY

In this PhD thesis, a droplet optofluidic diffraction grating is designed based on multiphase microdroplet technology; a droplet optofluidic system is developed to detect bacteriophages in water sample via host cell growth in microdroplet carrier and endothelial cell is cultured and its dysfunction under different physical and chemical conditions is measured using a hemodynamic microfluidic chip.

The reconfigurable optical diffraction grating using multiphase optofluidic droplets is designed based on a microdroplet system. The formation process of droplets in microfluidic T-junction geometries is described. The optical droplet grating works with high stability and tunability in grating period. Refractive index variation and various diffraction patterns are obtained. The grating is developed as a phase-transmission-grating-based compact optofluidic refractometer and zero-order transmission color filters, making it promising for biochemical and bio-material applications.

The droplet-based optofluidic system to detect bacteriophages is designed and fabricated. The sample containing bacteriophages is mixed with the host cell, *Escherichia coli*, and divided into a large amount of microdroplets. The growth of the host cell is monitored by the scattering pattern of the microdroplet carrier and the pattern is analyzed by using the mean power frequency. A real-time, label-free and high sensitivity quantification of bacteriophage is achieved and low concentration bacteriophage in water sample can be detected with high accuracy and stability.

Finally, a hemodynamic lab-on-a-chip microsystem is developed and used to study how hyperglycemia leads to the dysfunction of the endothelial cell. The endothelial cells are cultured in microfluidic chips under different physical and chemical conditions,

which can mimic the physiological pulsatile flow profile of blood vessel. The intracellular reactive oxygen species (ROS) level is determined using real-time fluorescence microscopy and the cell apoptosis is detected based on a caspase-3 based fluorescence resonance energy transfer (FRET) biosensor cell line which can detect endothelial cell apoptosis in real-time, post-treatment effect with limited cell sample.

The successful developments of living cell culture and detection are achieved using the microfluidic chip. The droplet grating shows a refractive index unit (RIU) detection limit of 6.3×10^{-5} when used as an optofluidic refractometer. It also can produces different colors when used as a color filter. The water sample with the bacteriophage concentration lower than 10^2 pfu/ml is detected and the result is in accordance with the calculated expectant. A more accurate assessment of the cell apoptotic percentage can be obtained with direct observation of fluorescence emission changes of the endothelial cell with biosensor cultured in the microfluidic chip.

Acknowledgement	i
Summary	iii
Table of Contents	v
List of Figures	ix
List of Tables	xvi
Nomenclature	xvii
Introduction	1
1.1 Motivation	1
1.2 Objective	6
1.3 Major contribution	8
1.4 Organization	12
2. Literature Survey	14
2.1 Droplet-based optofluidic devices	15
2.1.1 Microdroplet generation techniques	15
2.1.2 Microdroplet devices applications	19
2.1.3 Droplet-based optofluidic devices	22
2.2 Living cell analysis via droplet-based optofluidic devices	27
2.2.1 Droplet-based microfluidic technique for living cell analysis	27
2.2.2 Optical detection of living cell in microfluidic devices	31
2.2.3 Bacteriophage Detection and host cell detection	34
2.3 Endothelial cell and diabetes	37

2.3.1 Endothelial cell response to high glucose concentration.....	37
2.3.2 Endothelial cell response to physical stimulations	41
2.3.3 Endothelial cell dysfunction and death detection methods	44
2.4 Summary	47
3. Droplet Optofluidic Grating	48
3.1 Design of the droplet optofluidic grating	49
3.1.1 Multiphase microdroplets generation	49
3.1.2 Diffraction pattern analysis	55
3.1.3 Phase transmission optical properties.....	61
3.2 Fabrication and packaging	66
3.2.1 Fabrication processes.....	66
3.2.2 Fabrication and packaging.....	68
3.3 Experimental results and discussions.....	71
3.3.1 Multiphase microdroplet generation.....	73
3.3.2 Diffraction pattern	76
3.3.3 Droplet optofluidic refractometer	79
3.3.4 Droplet grating based transmission color selective system.....	82
3.4 Summary	86
4. Bacteriophage detection by droplet-based optofluidic system	88
4.1 Droplet-based microfluidic system for bacteriophage detection.....	89
4.1.1 Principle of droplet-based microfluidic method.....	89
4.1.2 Optofluidic system design and fabrication	92
4.1.3 Relationship between bacteriophage amplification and host cell growth	95

4.2 Droplet scattering pattern	97
4.2.1 Scattering pattern.....	97
4.2.2 Mean power frequency analysis	101
4.2.3 Principal component analysis	104
4.3 Bacteriophage detection via host cell growth	107
4.3.1 Co-culture of <i>E.coli</i> with λ bacteriophage	107
4.3.2 Co-culture of <i>E.coli</i> with M13 bacteriophage	109
4.3.3 λ bacteriophage detection	110
4.4 Summary	112
5. Endothelial cell dysfunction study using microfluidic chip	114
5.1 Hemodynamic Lab-on-chip system for Endothelial cell study	
5.1.1 Endothelial cell response to physical and glucose stimulations.....	115
5.1.2 Design of the microfluidic system.....	119
5.1.3 Control on the pulsatile flow condition	124
5.1.4 Endothelial cell culture in microfluidic chip	126
5.2 Endothelial cell dysfunction detection methods	127
5.2.1 Measurement of reactive oxygen species by H ₂ DCFDA	127
5.2.2 Mitochondrial morphological measurement.....	131
5.2.3 Endothelial cell death measurement using yo pro-1 and propidium iodide ..	133
5.2.4 Endothelial cell apoptosis measurement via FRET biosensor Caspase-3	137
5.3 Endothelial cell dysfunction detection	141
5.3.1 Measurement of reactive oxygen species overproduction.....	141
5.3.2 Measurement of mitochondrial morphological change	146

5.3.3 Measurement of endothelial cell apoptosis and necrosis	149
5.3.4 Inhibition of endothelial cell dysfunction using antioxidants	159
5.4 Summary	162
6. Conclusions	164
6.1 Conclusions	164
6.2 Recommendations	169
Author's Publications	171
Bioliography	173

LIST OF FIGURES

Fig. 3.1	Wettability of aqueous droplet: (a) On free surfaces and (b) In microchannels.....	50
Fig. 3.2	Schematic of a T-junction microchannel for droplet generation.....	51
Fig. 3.3	Types of diffraction gratings small slits: (a) Phase grating (b) Relief grating and (c) Generation of diffraction pattern orders after transmission through an array of.....	54
Fig. 3.4	(a) Schematic of the droplet optofluidic grating: (b) Top view of the droplet optofluidic grating and (c) Cross view of the droplet optofluidic grating.....	58
Fig. 3.5	Diffraction pattern analysis of the droplet grating: (a-b) Circular diffraction pattern of single droplet and (c-d) Vertical diffraction pattern of droplet array.....	59
Fig. 3.6	Angular spread of the first three orders of the spectrum for the droplet optofluidic diffraction grating.....	59
Fig. 3.7	The wave front phase shift profiles for different types of droplets.....	61
Fig. 3.8	Simulation results of the diffracted efficiency of the zero-order and first-order diffraction as a function of droplet refractive index.....	63
Fig. 3.9	Simulation results of the spectrum shift of the zero-order with four different droplet refractive indices.....	64
Fig. 3.10	Fabrication process flow of PDMS microfluidic devices.....	66
Fig. 3.11	Micrograph of the PDMS microfluidic chip.....	68

Fig. 3.12	Schematic of the microfluidic chip for droplet generation and 1-D droplet grating formation.....	70
Fig. 3.13	Schematic of the experimental setup.....	71
Fig. 3.14	Micrographs of the generation of droplets and the formation of droplet array.....	72
Fig. 3.15	The tuning of droplet size and grating period by microfluidic control: (a) Relationship between the size of single droplet and flow rate of the continuous oil phase and (b)-(e) Different droplet sizes and grating pitches achieved by tuning the flow rate.....	74
Fig. 3.16	Optical micrographs of the diffraction pattern of one-dimension droplet array with different grating period using monochromatic incident light: (a-c) Simulation results and (d-e) Experimental results.....	76
Fig. 3.17	Relationship between the single droplet diameter and circular pattern period.....	77
Fig. 3.18	Relationship between the grating pitch and vertical pattern period.....	77
Fig. 3.19	Relationship between the refractive index and the modified diffraction efficiency of 0 th and 1 st order.....	78
Fig 3.20	Diffraction pattern of 0 th and 1 st order of different droplet refractive indices.....	80
Fig. 3.21	The tuning of droplet size and grating period by microfluidic control: (a) Relationship between the size of single droplet and flow rate of the continuous oil phase and (b)-(e) Different droplet sizes and grating pitches achieved by tuning the flow rate.....	82

Fig. 3.22	Measured diffractions of white incident light when the droplet grating is used as a 0th order color filter: (a) Color patterns (b) SPD of light source and (c) Measured chromaticity values of the subtractive colors as compared to the predicted results.....	83
Fig. 4.1	Schematic illustration of optofluidic chip for bacteriophage detection that consists of: (a) Water sample containing bacteriophage and <i>E. coli</i> in culture medium are split into microdroplets by microfluidic structure, (b) Two microdroplets merge together and water sample is mixed with host cell <i>E. coli</i> , (c) Host cell growth condition in the microdroplet is affected by the presence of the bacteriophage and (d) Host cell growth condition is detected by using optical detection system.....	90
Fig. 4.2	Schematic of the optofluidic chip design.....	91
Fig. 4.3	Optical setup of the optofluidic system.....	92
Fig. 4.4	The transmitted optical density of <i>E. coli</i> culture individually or co-culture with λ phage and M13 phage.....	93
Fig. 4.5	Schematic illustration of the optofluidic imaging of droplets encapsulated with co-cultured bacteriophage and host cells, <i>E. coli</i>	96
Fig. 4.6	The scattering pattern of microdroplets containing different concentration of <i>E. coli</i> : (a) 10^9 cell/ml (b) 5×10^8 cell/ml (c) 2×10^8 cell/ml (d) 10^8 cell/ml (e) 5×10^7 cell/ml (f) 2×10^7 cell/ml (g) 10^7 cell/ml and (h) Control group.....	98
Fig. 4.7	Scattering pattern analysis with mean power frequency of microdroplets containing different concentration of <i>E. coli</i> : (a) The scattering pattern and intensity distribution of <i>E. coli</i> of 10^9 cell/ml (100 cell/droplet) and (b) The	

	scattering pattern and intensity distribution of <i>E. coli</i> of 10^7 cell/ml (0-1 cell/droplet)	100
Fig. 4.8	Mean power frequency changed by the <i>E. coli</i> concentration in the microdroplet.....	102
Fig. 4.9	Scattering pattern analysis with principal component analysis (PCA)	104
Fig. 4.10	The transmitted optical density of of <i>E. coli</i> culture	105
Fig. 4.11	The transmitted optical density of of <i>E. coli</i> co-culture with λ phage...	106
Fig. 4.12	The transmitted optical density of <i>E. coli</i> co-culture with M13 phage.....	108
Fig. 4.13	Signal recording of λ phage containing sample with the concentration of 10^3 pfu/ml and 10^4 pfu/ml.....	110
Fig. 5.1	Blood vessel structure and function of endothelial cells.....	114
Fig. 5.2	Schematic illustration of bio-model of endothelial cells interacts with their physical and biochemical microenvironments.....	116
Fig. 5.3	Schematic illustrations of: (a) The design of the microfluidic chip and (b) The hemodynamic Lab-on-a-chip system with configurable flow profile to mimic the blood flow in blood vessel.....	118
Fig. 5.4	Design of the microfluidic chip. Schematic illustration of: (a) Hemodynamic microfluidic chip system and (b) Experimental process to study endothelial cell death under different pulsatile shear stress conditions.....	121
Fig. 5.5	The volume flow rate profile of plasma in a typical artery with endothelial cells under a shear stress of 15 dyne cm^{-2}	123
Fig. 5.6	Phase images of endothelial cells: (a) Seeded in the microchannel and (b) Cultured in the microchannel for 48 hours.....	125

Fig. 5.7	H ₂ DCFDA is hydrolyzed inside the cell, retained in the cytoplasm, and then oxidized by ROS, leading to the emission of green fluorescence.....	127
Fig. 5.8	The fluorescent images of endothelial cell treated with H ₂ DCFDA at (a) Low ROS level and (b) High ROS level.....	128
Fig. 5.9	Mitochondrial morphology detection by MitoTracker green.....	130
Fig. 5.10	Fluorescent images of endothelial cells with (a) Filamentous reticular mitochondrial networks, and (b) Mitochondrial fragmentation.....	131
Fig. 5.11	Chemical structures of yo pro-1 and propidium iodide.....	132
Fig. 5.12	Measurement of cell apoptosis and necrosis by using yo pro-1 and propidium iodide: optical micrograph of apoptotic and necrotic fluorescing in and red, respectively.....	133
Fig. 5.13	Statistical analysis on the rate of apoptosis and necrosis under different glucose concentrations for 12 h. Etoposide is used as the positive control to test the effect of double-labeling method.....	134
Fig. 5.14	Principle of a living cell biosensor in detecting caspase-3 activation based on FRET effect: (a) Living HUVEC-C3 with a 535 nm green fluorescent emission and (b) Dead HUVEC-C3 with cleaved linker peptide and a 480 nm blue fluorescent emission.....	136
Fig. 5.15	Detection of caspase-3 activation in living apoptotic cells based on FRET effect. (a) Merged FRET image of HUVEC-C3 with live cells appeared in green color and apoptotic cells in blue color and (b) Statistical analysis of apoptotic rate after 24 h of 3 min UV-exposure, 500 nM of Taxol, and 20 mM of glucose treatments.....	139

Fig. 5.16	Time-dependent responses of the ROS level in endothelial cells under different shear stress profiles.....	141
Fig. 5.17	The ROS level in endothelial cells being exposed to 10 mM glucose with pulsatile shear stress of 15 dyne cm ⁻²	143
Fig. 5.18	The ROS level in endothelial cells being exposed to 10 mM glucose with pulsatile shear stress of 30 dyne cm ⁻²	144
Fig. 5.19	Mitochondrial morphology of endothelial cells under different shear treatment conditions for 3 hours.....	145
Fig. 5.20	Mitochondrial morphology of endothelial cells with 20 mM glucose under different shear treatment conditions.....	147
Fig. 5.21	Measurement of cell apoptosis and necrosis by using yo pro-1 and propidium iodide, (a) Statistical analysis on the rate of apoptosis and necrosis under different glucose concentrations with a flow profile of 2 h SS 30 (30 dyne cm ⁻²) and 10 h SS15 (15 dyne cm ⁻²) and (b) Statistical analysis on the rate of apoptosis and necrosis different flow profiles conditions with 20 mM glucose. Sample size: 1,000 cells.....	150
Fig. 5.22	Detection of caspase-3 activation in living apoptotic cells based on FRET effect. Statistical measurements of percentage of apoptotic cells using caspase-3 activation under different glucose concentrations and different flow profile conditions. Sample size: 1,000 cells.....	151
Fig 5.23	FRET changes in response to caspase-3 activation with different glucose concentrations (a) 5 mM (b) 10 mM and (c) 20 mM under shear stress 30 dyne cm ⁻² 2 h plus shear stress 15 dyne cm ⁻² 10 h.....	155

Fig. 5.24	FRET changes in response to caspase-3 activation under different conditions (a) 5 mM glucose under shear stress of 15 dyne cm ⁻² for 12 h, (b) 20 mM glucose under shear stress of 15 dyne cm ⁻² for 12 h (c) 20 mM glucose under shear stress of 30 dyne cm ⁻² for 1 h plus shear stress of 15 dyne cm ⁻² for 11 h and (d) 20 mM glucose under shear stress of 30 dyne cm ⁻² for 2 h plus shear stress of 15 dyne cm ⁻² for 12 h.....	156
Fig. 5.25	Time-dependent responses of (a) ROS level, (b) Mitochondrial damage and (c) Caspase-3 activation and cell apoptotic rate are measured by yo pro-1 with glucose concentration of 20 mM and different flow conditions.....	157
Fig. 5.26	Effect of propyl gallate (PG) and butylated hydroxyanisole (BHA) on shear-induced increase of ROS production in endothelial cells.....	158
Fig. 5.27	Effect of antioxidant drugs on high glucose and shear stress induced cell apoptosis.....	160

LIST OF TABLES

Table 3.1	Relationship between concentration and refractive index of Calcium Chloride Solution.....	54
Table 3.2	The chemical and physical properties of PDMS.....	67

NOMENCLATURE

LOC	Lab-on-a-chip
DEP	Dielectrophoresis
EWOD	Electrowetting on dielectric
RIU	Refractive index unit
DNA	Deoxyribonucleic acid
FACS	Fluorescently activated cell sorter
OFM	Optofluidic microscopy
DAG-PKC	Diacylglycerol protein kinase C
ROS	Reactive oxygen species
NADPH	Nicotinamide adenine dinucleotide phosphate
FRET	Fluorescence resonance energy transfer
FFT	Fast Fourier Transform
PCA	Principal component analysis
PDMS	Polydimethylsiloxane
CAD	Computer-aided design
IPA	Isopropanol solution

CHAPTER 1

INTRODUCTION

1.1 Motivation

This PhD research topic is motivated by the potential of optofluidic technology in exploring the development of living cell detection and culture. Optofluidics, usually defined as the combination of optics and fluidics, takes advantage of the unique properties of liquid for the purpose of optical applications. The introduction of microfluidics into optical devices enables the designer to modify the optical properties of the devices, to obtain smooth surfaces or interfaces between optical media, and dynamically reconfigure the shape of optical components [1, 2]. The optofluidic technology has made possible integration of optical-based and chemical-based biological detection into microsystems low cost, high sensitivity, high reliability and stability [3-6]. In this PhD research topic, three optical-based and chemical-based biological detections are designed and realized. A droplet optofluidic diffraction grating, which has the applications as refractometer and color filter, represents the optofluidic-based chemical measurement. Then a droplet optofluidic system is developed to detect bacteriophages in water sample via host cell growth in microdroplet carrier, which shows the performance of optofluidic system in the area of biological sample measurement. Furthermore, the endothelial cell is cultured and its dysfunction under different physical and chemical conditions is measured using a hemodynamic chip. The bio-sample such as living cell is integrated within the optofluidic system and the culture and observation are developed. In

this PhD research topic, the ability of optical detection combining with multiple microfluidic techniques such as microfluidic and cell culture in microchip are showed and the optofluidic application in different biological areas are demonstrated.

A multiphase optofluidic system can also prevent a liquid from direct contact with microchannel walls and thereby eliminate or reduce undesired clogging of channels and contamination. An individual droplet is ideally suited to compartmentalizing and isolating small samples. The significant challenge encountered in single-phase microfluidic systems is being able to suppress dispersion of reacting volumes. Under typical conditions, channel walls impart shear forces on any contained fluid, therefore, under hydrodynamic pressure a parabolic velocity profile is established over the cross-section with fluid velocity zero at the channel walls and maximum at the channel center [7-9]. This generates a residence-time distribution that can cause significant variation in the yield, efficiency and product distribution of a given reaction. Accordingly, localization of reagents within discrete droplets is an effective way of eliminating this phenomenon and allowing precise definition of reaction or incubation times in a micro-bioreactor [10, 11]. This precision combined with high droplet throughput and well-defined droplet size renders microdroplet technology an ideal platform for the quantitative readout of a particular process. The droplet generation in a rapid and reproducible fashion is crucial for the ultimate success of an experiment [12-15]. At last, droplet microfluidics also offers greater potential for increased throughput and scalability than continuous-flow systems. Although single-phase continuous flow systems offer fine control over flow characteristics, scaling up is a challenge as the size of devices scale almost linearly with the number of parallel experiments. Droplet microfluidics, however,

has the ability to perform a large number of reactions without increasing device size or complexity [10, 11, 16].

Based on the latest Contaminant Candidate List (CCL) by US Environmental Protection Agency, out of 12 microbial contaminants, 4 are viruses, which are key human pathogens that cause respiratory illness, gastrointestinal illness and liver disease. Viruses live in untreated water, treated water, tap water and swimming pool. They are significantly smaller than bacteria and cells and hence can easily escape from filtration barriers. Besides, some viruses, such as adenoviruses, are more resistant to water treatment including UV disinfection. Therefore, drinking water sources have to be constantly monitored to avoid viral contamination in order to reduce human health risk. The conventional methods usually require pre-concentration and culture assay or molecular biological assay, which do not allow real-time detection within a shorter time period. In this proposal, a droplet-based optofluidic system is realized to monitor and detect bacteriophages in drinking water. The optofluidic system provides new potentials in the monitoring and detection of bacteriophages in a single chip with real-time, high accuracy, high sensitivity and low cost. Bacteriophages are viruses that infect and replicate within bacteria. Bacteriophages are widely distributed in locations populated by bacterial hosts, such as soil or the intestines of animals. Waterborne viral outbreaks are still one of the most serious problems in drinking water quality monitoring [17-19]. To reduce human health risks from viral infection, drinking water sources have to be constantly monitored to avoid viral contamination. The concentration of bacteriophages is significantly correlated with virus concentrations in water samples [20]. The use of phages as surrogate indicator for viruses has been applied in the routine monitoring of

raw and treated drinking water supplies and in the assessment of the efficiency of domestic point-of-use water treatment units [21].

Current available bacteriophage detection techniques are adapted from the clinical applications. The main challenge in their detection is the low concentration of bacteriophage in drinking water samples. A bacteriophage pre-concentration step is necessary before target detection can be achieved. In addition, the sensitivity of the detection technique is critical to identify the target. The bacteriophage concentration step is performed based on the membrane filter adsorption and elution. Common detection techniques include plaque assay method using *E. coli* as host and molecular biology based assay such as polymerase chain reaction (PCR) methods [22-24]. The limitations of these techniques include low recovery rate from membrane filtration, long processing time for culture assay and low efficiency in PCR due to the presence of inhibitors in water matrices. It is difficult to realize rapid and sensitive monitoring and detection of bacteriophages in water samples.

The endothelial cells line the internal lumen of all vasculature and serve as an interface between circulating blood and vascular smooth muscle cells, functioning as a physical barrier between blood and tissues and facilitating a complex array of functions in intimate interaction with the vascular smooth muscle cells. The endothelial cells also have a critical role in overall homeostasis whose functions are integrated by a complicated system of chemical mediators [25]. The ability of the blood vascular endothelial cell to sense and respond to the flow of blood is observed [18]. Changes in blood flow, thus generating altered hemodynamic forces are responsible for acute vessel tone regulation, the development of blood vessel structure during embryogenesis and

early growth, as well as chronic remodeling and generation of adult blood vessels. The complex interaction of biomechanical forces, and more specifically shear stress, derived by the flow of blood and the vascular endothelium is still not well studied [19, 20].

Under normal physiological circumstances, there is a balanced release of the endothelial cell derived relaxing factors such as nitric oxide (NO) and prostacyclin (PGI₂), and contracting factors such as endothelin-1 (ET-1), prostaglandins, and angiotensin II (ANG-II). Diabetes is a metabolic disorder which is characterized by hyperglycemia and glucose intolerance due to insulin deficiency, impaired effectiveness of insulin action or, both. The dysfunction of the endothelium is regarded as an important factor in the pathogenesis of vascular disease in diabetes mellitus [25-27]. In endothelial cell dysfunction, this balance is destroyed [26]. The dysfunction of endothelium in diabetes mellitus is characterized by changes in proliferation, barrier function, adhesion of other circulating cells, and sensitivity to apoptosis. Furthermore, diabetes mellitus is found to modify the angiogenic and synthetic properties of endothelial cells [27, 28]. Apoptosis is a controlled and regulated process that involves individual cells and is a normal component of the health development of multicellular organisms and continues throughout adult life. The role of endothelial apoptosis in diabetes mellitus is very complicated, due to many independent factors such as ageing, obesity, hyperlipidemia, hypertension, low grade inflammation, insulin resistance and hyperglycemia. However, the relationships between endothelial dysfunction and these factors are still not completely understood despite a lot of research because of the complex coefficients among the individual factors [25].

In summary, optofluidic techniques provide various applications in establishing optical systems with low-cost fabrication, high flexible tunability and high detection accuracy. On the other hand, the droplet-based optofluidic facilitates as a powerful tool for physical self-pattern unit, cell culture and manipulator. The integration of optofluidic techniques with microdroplet explores the possibility for new optofluidic system design and cell studies, which is chosen as the topic of this thesis.

1.2 Objective

The main objective of this research is to design an optofluidic chip with a microdroplet system for living cell culture and detection with convenient operation and high measurement precision. The research investigations include theoretical analysis, fabrication process development and experimental studies of the optofluidic chip. The theoretical models and analysis of the microdroplet-based grating include microstructure formation and optical properties calculation. The microfluidic design is integrated into the chip to facilitate cell manipulation and detection. The design is also integrated to mimic as a real biological design using buffer modulation. A soft-lithography process is also developed for the fabrication of the optofluidic chip.

A novel droplet optofluidic grating is designed. The unique features of the microdroplets such as uniformity, periodicity and self-assembly, make them particularly suitable for optical gratings. In this work, the dynamical and reversible tunability and stability of the droplet grating will be optimized by controlling the microdroplets formation system. Various applications of the droplet optofluidic grating are also developed. The droplet array grating can work as a phase-transmission-grating-based compact optofluidic refractometer as well as a zero-order transmission color filter.

The droplet optofluidic detection system is capable of detecting and quantifying bacteriophages effectively. A water sample with low concentration of bacteriophages is divided into multiple microdroplets after mixing with host cells. With the small volume confinement and high contact ratio, the effective concentration of bacteriophages increases and the infection induces significant influence to host cells. The host cells growth can be monitored by a light scattering system which realizes a real-time, label-

free and high sensitivity detection. The optical signal of large amount of single microdroplets can be analysed fast and accurately by developing a new measurement method to detect very few bacteriophages in a large volume of water.

In order to study hyperglycemia-induced endothelial cell dysfunction, the study proposes a highly controllable microfluidic chip for detecting the cellular responses under different combinations of physiological pulsatile shear stresses and high glucose concentrations. Shear stress is directly imposed onto the endothelial cells' surface and high concentration of glucose is added into culture medium to mimic the physical and chemical stresses applied to the cells. This system is biocompatible with a highly controllable microfluidic platform such that cells can be cultured in the microchip with the controlled perfusion of culture medium, and different buffer media or dye solutions can be easily injected into the microchip under predefined flow rates. A novel biosensing assay based on caspase-3 activity, which can detect apoptosis-activating compounds in a high throughput manner with a genetically engineered cell line, is integrated into the platform.

In summary, in this PhD research, living cell detection and culture are achieved using the microfluidic chip. A droplet optofluidic grating is designed and fabricated for various optical applications. Water sample containing bacteriophage with low concentration is monitored and bacteriophage is detected. The accurate cell apoptosis detection is achieved using a biosensor combined with microfluidics chip mimicking the real physical microenvironment.

1.3 Major contribution

The major contributions of this PhD thesis lie in various aspects of the theoretical analyses, designs, fabrications, and experimental verification of the three different optofluidic systems for living cell detection and culture. The details are as follows:

- 1) The formation process of droplets in an optofluidic grating using T-junction geometries microchannel is described. At low capillary numbers, the dominant contribution of the dynamics break-up arises from the pressure drop across the emerging droplet. A simple scaling relation predicts the size of droplets produced in T-junctions over a wide range of flow rates of the two immiscible phases, of the viscosity of the continuous phase, of interfacial tension and of geometrical dimensions of the device. (See Chapter 3)
- 2) A reconfigurable optical diffraction grating using multiphase droplets is designed on an optofluidic chip. The uniform and evenly spaced circular droplets are generated by continuously dispersing two immiscible liquids into a T-junction to produce plugs, which are then transformed into a circular shape at a sudden expansion of the microchannel. The droplet optofluidic grating shows a detection limit of 6.3×10^{-5} when it is used as an optofluidic refractometer and produces different colors as a color filter. (See Chapter 3)
- 3) A droplet optofluidic detection of bacteriophages through measuring the growth of the host cell infected by bacteriophages in a microdroplet carrier is designed and fabricated. The growth of the host cell, *Escherichia coli* (*E. coli*), is detected by the scattering pattern of the microdroplet carrier and the pattern is analyzed using mean power frequency. Based on the relationship between the mean power

frequency and the concentration of the *E. coli* in the microdroplet carrier, the increment of a single *E. coli* can be detected between 0 to 20 *E. coli* cells in one droplet while the increment of 2 *E. coli* can be detected from 21 to 100 *E. coli* cells. (See Chapter 4)

- 4) The growth curve of *E. coli* cultured individually or co-cultured with λ bacteriophage or M13 bacteriophage is further portrayed using the previously mentioned detection method. The λ bacteriophage-containing water sample is measured using the droplet-based optofluidic system, and a limit-of-detection of 10^3 pfu/ml and 10^4 pfu/ml of the bacteriophage is measured and found to be in accordance with the calculated theoretical value. (See Chapter 4)
- 5) A hemodynamic microfluidic chip system is developed for better understanding how hyperglycemia induces endothelial cell dysfunction under diabetic conditions. The system is designed to mimic the physiological pulsatile flow profile in blood vessel. The endothelial cells performance under physiological pulsatile shear stresses and different glucose concentrations are investigated. (See Chapter 5)
- 6) The intracellular ROS level is studied using real-time fluorescence microscopy with the measurement of the oxidation of 2', 7'-dichlorodihydro-fluorescein diacetate by hydroxyl radicals or hydrogen peroxide molecules. The mitochondrial morphology is studied via fluorescent MitoTracker staining. The shear-induced cellular responses of endothelial cells under different glucose concentration are studied by mimicking the physiological pulsatile flow profiles in blood vessel. The results show that ROS level is elevated during exhaustive

- exercise (shear stress of 30 dyne cm^{-2}) and with high glucose concentration (diabetes patient). (See Chapter 5)
- 7) The hemodynamic system combines a caspase-3 based fluorescence resonance energy transfer (FRET) biosensor cell line which can detect endothelial cell apoptosis in real-time, post-treatment effect and limited cell sample. A double-labeling fluorescent technique (yo-pro-1 and propidium iodide) is used to validate the findings revealed by the FRET-based caspase sensor. A 12% apoptotic rate (nearly 4-fold as compared with the static condition) is observed when the endothelial cells are exposed to a high glucose concentration of 20 mM under 2-hour exhaustive pulsatile shear stress of 30 dyne cm^{-2} and followed by another 10-hour normal pulsatile shear stress of 15 dyne cm^{-2} .

1.4 Organization of the thesis

The thesis is organized into six chapters. The introduction of the thesis covers the motivation, objective, and major contributions of the thesis as presented in this chapter. The motivation section explains why and how the Ph.D. research is carried out. The objective states the main focus of this thesis, and the contribution section lists the innovations and important findings in both the theoretical and technological aspects.

In Chapter 2, a literature review of optofluidic microdroplet-based technique for living cell culture and detection is performed. This chapter reviews the design and applications of droplet-based optofluidic devices. Then, the state-of-the-art of the droplet optofluidic devices for living cell analysis is discussed, especially of the relationship between host cell and bacteriophage. The review also covers the background work of endothelial cell dysfunction and the detection of endothelial cells under different conditions.

In Chapter 3, the design, fabrication and experimental testing of an optical diffraction grating using multiphase microfluidic droplets are presented. It employs only one row of droplets and optical illumination from the top without the need for liquid waveguide and is, thus, advantageous over the reported optofluidic grating designs in aspects of simple structure, easy implementation, high sensitivity, improved tuning speed, high uniformity, and high stability.

In Chapter 4, the design, fabrication and experiments of a droplet-based optofluidic system for λ bacteriophage and M13 bacteriophage detection are presented. The bacteriophage is co-cultured with *E. coli*, the host cell, and then divided into large

number of droplets. The *E. coli* growth in the microdroplet carrier is influenced by the infection of the bacteriophage, which results in the change of the concentration of *E. coli*. The concentration of *E. coli* in the microdroplet is measured by the scattering pattern of the droplet using mean power frequency value. Water samples containing different concentrations of λ bacteriophage are then measured using the optofluidic system. The measurement results agree with the analysis resulted from the theoretical calculations.

In Chapter 5, the design, fabrication and experimental testing of a novel endothelial cell dysfunction detection hemodynamic system, which combines a caspase-3 based FRET biosensor cell line and the microfluidic chip system, are elaborated. The intracellular ROS level is determined using real-time fluorescence microscopy and the mitochondrial morphology is studied via fluorescent MitoTracker staining. The apoptosis and necrosis of endothelial cells undergo a series of different pulsatile flow conditions and glucose concentrations can be detected with high accuracy since the FRET biosensor provides real-time observation and do not need any post-treatment.

Chapter 6 concludes and summarizes the major contributions of this thesis followed by the recommendations for future work.

CHAPTER 2

LITERATURE SURVEY

This chapter consists of three parts. The first part presents a literature review of on various microdroplet-based optofluidic devices. The review focuses three aspects reported in literature: a) The development of optofluidic devices, b) The studies of the microfluidic based droplet devices and c) The various applications based on the combination of microdroplet techniques with optofluidic detection. The second part focuses on living cell manipulation and analysis via droplet-based optofluidic devices. The techniques of optofluidic design for living cell measurement and the microdroplet carrier for living cell manipulation are recommended. In the third part, the results of the research on the dysfunction of endothelial cell related to blood vessel damage in diabetes patients are introduced. Traditional detection methods that look into the influence of blood glucose concentration and physical stimulations on endothelial cell growth and dysfunction are presented.

2.1 Droplet optofluidic devices

2.1.1 Microdroplet generation techniques

As interest grows in droplet-based microfluidics, more technologies are being developed for the generation and control of droplets. Although microdroplet flows driven by surface and interfacial tension gradients have been studied over the last century, it is only within the last few years that the microfluidics community has renewed its interest in microdroplets and their applications.

In recent years, microfluidic systems have been used to generate multiphase fluids in a variety of formats. Although microdroplets may be formed in microchannels under static conditions [29], the adoption of continuous or pseudo continuous flows has been shown to provide the most versatile route to high-throughput droplet generation. A key advantage of using the microfluidic systems is the ability to significantly reduce droplet size distributions when compare to bulk methods. Moreover, the droplet production can be parallelized to increase the throughput and cross-contamination between adjacent microdroplets can be essentially eliminated.

The generation of microdroplets within microfluidic channels can be initiated through the use of electric fields [30-32]. Emerging technologies have enabled electrodes to be integrated into microdevices to provide electrical control over droplet formation. Two examples are dielectrophoresis (DEP) and electrowetting on dielectric (EWOD) [33, 34]. The operation principle behind DEP-driven droplet formation is based on the phenomenon that polarize fluids will be attracted to areas of higher electric field intensity. The mechanism of EWOD-based generation relies on the observation that an electric field can change the interfacial energy between a fluid and the contact surface.

Besides using electric fields, other methods such as micro-injectors and needles are also widely applied. For the former category micro-injectors, individual droplets are generated on-demand using a microfluidic chamber. The optical vortex traps are implemented to manipulate and transport the generated droplets, which have a lower refractive index than the immiscible medium in which the droplets are immersed and thus cannot be trapped using conventional optical tweezers [35]. For the latter category, simple flexible tubing and narrow gauge needles can be used to replace classic elastomeric and hard material microfluidic devices. The design yields laminar, transitional, droplet, and chaotic phases in the same way as classic devices and has the added advantage that both the tubing and needle are tubular [36].

However, of particular interest are the microfluidic systems that exploit flow instabilities between immiscible fluids to generate suspended droplets. Droplets can spontaneously form when laminar streams of aqueous reagents are injected into an immiscible carrier fluid. The two most commonly used methods for generating droplets in this way are through the use of flow focusing and T-junction geometries. A number of focused reviews provide detailed insight into droplet generation and flow dynamics [37-39]. Hydrophobic channel surfaces are required to prevent unstable, polydisperse water-in-oil droplet formation due to wetting effects [40]. In the flow focusing configuration, the dispersed and continuous phases are forced through a narrow region in a microfluidic channel. The design employs symmetric shearing by the continuous phase on the dispersed phase which enables more controlled and stable generation of droplets. The continuous phase applies pressure and viscous stresses that drive the dispersed phase into a narrow strand, which then breaks inside or downstream of the orifice to form droplet

[40-43]. For flow-focusing nozzles, water-in-oil droplets break off from the aqueous fluid stream when oil shear stress overcomes interfacial tension that keeps droplets attached to the aqueous neck. Droplet size and formation rates depend on the combinations of aqueous and oil-phase flow rates, nozzle geometry, respective dynamic viscosities μ and densities ρ and interfacial tension between phases [44].

In the T-junction configuration, the inlet channel containing the dispersed phase perpendicularly intersects the main channel which contains the continuous phase. The two phases form an interface at the junction, and as fluid flow continues, the tip of the dispersed phase enters the main channel. The shear forces generated by the continuous phase and the subsequent pressure gradient cause the head of the dispersed phase to elongate into the main channel thinning the neck of the dispersed phase and eventually break the stream into a droplet. For T-junction nozzles at low oil flow rates, the oil flow ‘pinches’ droplets when the aqueous stream blocks the continuous flow channel, thereby creating a pressure which leads to droplet generation [45]. In T-junctions, slightly different breakup mechanisms lead to dripping/jetting transitions. The aqueous fluid interface moves into the continuous fluid stream and creates a blockage in the channel and the upstream pressure builds until the pressure force overcomes the interfacial tension forces, and the droplet is ‘pinched’ off the main stream. Similar to the dripping regime for flow-focusing and co-flowing nozzles, monodisperse droplets form as the oil shear pulls droplets from the main aqueous stream [44, 46]. Recent experimental pressure measurements in T-junctions also have found that pressure oscillations still persist even in the dripping regime, suggesting that the dripping mechanism may not be entirely

shear-driven. The size of the droplets can be changed by altering the fluid flow rates, the channel widths, or by changing the relative viscosity between the two phases [47-50].

To ensure steady droplet generation in flow focusing or T-junction schemes, aqueous and oil flow rates must therefore be sufficiently small to avoid inertial and shear-based jetting. This imposes a strict throughput limitation on the attainable flow rates and drop generation frequencies for a given nozzle. Thus, increasing throughput must be attained by adding parallel channels and not by simply increasing flow rates [44, 51, 52].

2.1.2 Droplet-based microfluidics

In nature, chemical and biological operations are carried out in micro-sized spaces such as in cells and their organelles. Droplet microfluidics offers the capability to form femto- to picoliter sized droplets and to compartmentalize and mimic reactions and molecular process within individual droplets. Both immiscible individual fluids separated by interfaces and the interfaces themselves provide us with mediums that are very suitable to study microreactions and interfacial phenomena. Microdroplets are almost ideal chemical reactors characterized by fast thermal transfer efficient mixing, narrow residence time and absence of hydrodynamic dispersion. At the core of droplet microfluidics are the fundamental transport equations for mass, momentum and energy conservation. When increasing flow rates to increase device throughput, one necessarily must consider and quantify the effects of pressure and shear forces on droplets and encapsulated samples, especially for biological applications.

Microdroplets separated by interfaces will confine substrates of interest inside and thus can act as microreactors that perform reactions in the microscale with significant advantages over their macroscopic counterparts: conserve expensive and precious reagents by operating with very small volumes, reduce exposure to hazardous chemicals and allow multiple reactions to be carried out in highly parallelized experiments. For aqueous droplets in immiscible oil carrier fluids, delivery of reagents and other solutes to and from cells is prevented by the low solubility of the aqueous phase in the oil phase. Droplets can be separated from channel walls by a thin layer of immiscible liquid which can transport products and prevent the accumulating of solid product. Yield increases with decreasing droplet size due to the increase of the specific area of the droplets. A

number of small-molecule reactions have been performed in liquid plugs within microfluidic systems. Since rapid mixing of the contained fluids is efficient at creating a homogeneous reaction environment, droplet-based microfluidic systems are ideally suited for use in kinetic studies [53]. Microdroplets systems also provide a versatile platform for use in protein crystallization, as typical droplets have nanolitre volumes. The effect of mixing on the nucleation of protein crystals is studied to elucidate the process of protein crystallization inside droplets [54-56]. A droplet-based microchannel chip has many advantages for synthetic reactions. In particle synthesis, in particular, it is possible to tune the sizes and size distributions of synthesized particles in a controllable fashion by varying linear velocity and mean residence time. Flow profiles and patterns influence the synthesis reactions [56, 57].

Mixing is an important tool required to carry out and study the kinetics of biological and chemical reactions. Rapid mixing is essential for applications where reactions, measurement and actuation must take place on millisecond or faster time scales, and where interrogation times in high-throughput devices are limited. Under laminar flow conditions, when two fluid streams come into contact with each other, there is not turbulent mixing and the only mixing behavior is diffusive. Although the diffusion distance is small, the time required to completely mix the two fluids remains long. However, when a droplet moves through a straight channel, an equal recirculating flow is generated in each half of the droplet that touches the channel wall [58]. To enhance internal mixing within droplets, channel geometry is used to create chaotic advection to fold and stretch the droplet content. The mixing inside droplets can also be electrically controlled. The droplets may be immersed in air or oil, and not in direct contact with the

electrodes but separated by a hydrophobic dielectric layer instead. The manipulation of droplets composed of water, buffers and biological fluids in both air and oil has been demonstrated [59, 60].

Microdroplets also have been shown to be powerful tools in the study of biology. There is a clear potential to study *in vitro* evolution of proteins and RNAs, cell-free cloning and sequencing, genetics, genomics and proteomics [61, 62]. The power of droplet microfluidics in measuring enzyme kinetics is also demonstrated. Using such an approach, single turnover kinetics of enzyme ribonuclease on a millisecond timescale is measured. Using several aqueous inlet channels with each of them supplying a different substrate for the reaction, it is possible to alter the substrate and enzyme concentration through a variation in flow rate ratios. Similarly, the enzymatic turnover by alkaline phosphatase and the activity of luciferase within droplets have been investigated [63, 64].

2.1.3 Droplet optofluidic detection

Optofluidics refers to a class of optical systems that are synthesized with fluids. It fundamentally aims at manipulating fluids and light at microscale and exploiting their interaction to create highly versatile systems. Most optical systems are currently made with solid materials such as glasses, metals and semiconductors, but there are cases in which it has been advantageous to use fluids. Fluids have unique properties such as the ability to change the optical property of the fluid medium within a device by simply replacing one fluid with another; the optically smooth interface between two immiscible fluids; and the ability of flowing streams of miscible fluids to create gradients in optical properties by diffusion. Integration and reconfigurability are two major advantages associated with optofluidics, whereas microfluidics has made it possible to integrate multiple fluidic tasks on a chip. The second advantage lies in the ease of changing the optical properties of the devices by manipulating fluids [2].

In general, optofluidic devices can be categorized into three major groups: (1) structures solid-liquid hybrids in which the optical properties of both media are relevant [71-74]; (2) colloid-based systems in which manipulation of solid particles in liquid, or using the unique optical properties of colloidal solution [75-77] and (3) complete fluid-based systems in which only the optical properties of the fluids are relevant [78-81].

The replacement of the solid substrate with a liquid in an optofluidic system can result in an even greater degree of flexibility [2]. Purely fluidic systems have several unique advantages that cannot be obtained from solid-fluid hybrids. First, the physical profile of the fluidic system that is optically relevant can be quite independent of the actual profile of the underlying microfluidic system. Second, it is possible to achieve

finer features in the composite fluid media. Finally, the interface between the two fluids can be made optically smooth by either choosing two immiscible fluids or by flowing the two fluids next to each other at low Reynolds numbers. The roughness of the underlying microfluidic architecture has little impact on the fluid interfaces. A liquid core liquid cladding optical waveguide which consists of an optically dense fluid flowing within an enveloping sheath of fluid with lower refractive index is an excellent example of such system [84]. A tunable optofluidic waveguide dye laser utilizing two centrifugal Dean flows. The definition of Dean flow is a kind of flow condition with very low Dean number. The Dean number is a dimensionless number giving the ratio of the viscous force acting on a fluid flowing in a curved pipe to the centrifugal force, which equal to the Reynolds number times the square root of the ratio of the radius of the pipe to its radius of curvature. [85] In this design a centrifugal Dean flow increases the light confinement of the dye laser by shaping a three-dimensional (3D) liquid waveguide from curved microchannels is further demonstrated. The demonstrated slope efficiency is at least 3-fold higher than its traditional two-dimensional equivalent.

On the other hand, the optical smoothness of fluid interfaces can be a useful and cost-effective way to create surfaces of similar quality in solid systems. A tunable liquid microlens using three laminar flows injected into an expansion chamber is designed. Different lens shapes and curvatures can be achieved and tuned through the control of three flow rates and the expansion chamber is designed to improve the fluidic stability and maintain the ideal lens shape for precise microscale optical measurement [86]. Another example is a tunable optofluidic prism based on the configuration of two laminar flow streams with different refractive indices in a triangular chamber. The chambers with

70° and 90° apex angles are designed based on simulation results, which provide the optimum working range and avoid recirculating flows in the chamber [87].

The use of immiscible fluids in microfluidic provides an additional route to periodic, self-assembled geometries, with the size of the discrete fluid elements and the volume fraction of the dispersed phase as easily tunable parameters. The initial application is the combination of microdroplet and micro-optics to form an optofluidic sensor for measuring dynamic interfacial tensions. The sensor is able to measure both surface tension and liquid-liquid interfacial tension because the formation frequency of the microbubbles or microdroplets is a function of the interfacial tension between the two phases. This frequency can be measured easily by optical detection [65].

Another important field of optofluidics is micro-droplet based lasers. A microdroplets a natural liquid and optical resonator that supports whispering gallery modes (WGMs), which circulate along the droplet surface and provide optical feedback [66]. Recently, rapid generation of a large number of micro-droplets and subsequent demonstration of using the corresponding droplets lasers using microfluidics with two immiscible liquids has been reported [67-69]. In particular, water-based droplets are important for bio/chemical applications [70]. An optofluidic ring resonator laser based on micro-bubbles filled with liquid gain medium is demonstrated. Due to the sub-micron wall thickness of the micro-bubble, a significant amount of the electric field resides inside the liquid. The device has 3-dimensional optical confinement, extremely high Q-factors [71]. A novel nL-sized microdroplet laser based on a capillary optofluidic ring resonator (OFRR) is also developed. The tuning of the lasing wavelength is achieved via

highly efficient fluorescence resonance energy transfer (FRET) by merging two different dye droplets in the microfluidic channel [72].

One property of liquids which may also be employed is optical absorption. Since the optical attenuation of a liquid can easily be altered by the addition of dyes or by varying the optical path length, many devices which use liquids as variable optical absorbers has been developed [73]. Miniaturized optical switches demonstrate particularly great potential applications. Recent work on optofluidic switches has employed a transparent droplet of glycerol placed between two glass substrates and surrounded by an opaque liquid phase. By deforming the liquid–liquid interface using dielectric forces, the droplet could be forced to wet both substrates, thereby opening a transparent channel between the substrates [74]. The same operating principle is employed in another study , where the robustness of the design is improved by using an aqueous droplet and electrowetting on dielectrics as the actuation mechanism [75].

Microdroplet techniques combined with optofluidic also enabled a new way to fabricate a real time controlled tunable grating. Optical diffraction gratings are typically fabricated by using solid materials such as glass, quartz, polymers or metals. Fluid-based optical components represent a class of components that is intrinsically well suited for dynamic, reversible control over optical properties. The adjustable parameters include both the geometry of the active components and the property of the material of which they are made. A periodic refractive index grating in optical waveguides is formed by microfluidic plugs infused into the air holes of microstructure optical fibers. These microfluidic resonant structures provide an alternative method for creating and tuning long-period gratings in optical fibers. Another report is about the realization of liquid

tunable long-period grating using multiphase droplet microfluidics. It provides tunability in the grating period by tuning the flow rates of the liquids, the refractive index and the index variation of the core layer by using different combinations of liquids [76]. Furthermore, a two-dimensional grating was realized based on a microfluidic platform comprising a flow-focusing bubble generator and flowing, regular lattices of bubbles formed by dynamic self-assembly. This diffraction grating exhibits high stability over hours of operation if properly designed and operated [77].

2.2 Living cell detection via droplet optofluidic

2.2.1 Droplet technique for living cell analysis

Droplet-based microfluidics involves producing microscale droplets of one fluid within a second immiscible carrier fluid. Recently, microdroplet devices have developed varied methods for high-throughput living cell manipulation and analysis. The advantages of droplet-based techniques include: confinement of living cells into microdroplet within a continuous phase, which provides isolation of cells by creating individual microreactors, thereby avoiding dilution of products, improving detectable signals and reducing reagent volumes. Moreover, the large droplet surface area to volume ratios can be exploited for enhanced heat and mass transport [44].

High-throughput encapsulation of cells into microdroplets benefits from the high droplet generation rates. To control cell encapsulation, a number of approaches may be utilized. Encapsulation may use a cell-triggered Rayleigh–Plateau instability in a flow-focusing geometry, and self-sorting employing two extra hydrodynamic mechanisms has achieved on-flight in continuous flow at a rate up to 160 cells per second [78]. Additional studies report methods to not only encapsulate single cells, but to repeatedly capture a number of cells to study both isolation and the interactions between cells in groups of controlled sizes. This study shows a single-particle encapsulation efficiency $P(k=1)$ of 83.7% and a double-particle encapsulation efficiency $P(k=2)$ of 79.5% as compared to their respective Poisson efficiencies of 39.3% and 33.3%, respectively [79]. To overcome the Poisson statistical limitation for random encapsulation, another study utilized densely packed gel particles in aqueous flows upstream of the flow-focusing droplet nozzle to encapsulate single gel particles at 1.5 kHz rates with 98% efficiency. It allows the

number of particles encapsulated, the size and volume fraction of the drops in which they are encapsulated, and the frequency and flow velocity of the drops to each be controlled independently [85]. However, adopting of the method to cells would require prior encapsulation of cells in gel particles to alleviate fluid stresses associated with flow-based close packing. In addition to aqueous droplet formation, agarose and other polymer hydrogels have been employed to encapsulate and immobilize cells or individual molecules within monodisperse microspheres. In these applications, particle composition is carefully selected to control the microcapsule's mechanical, diffusive and degradation properties. In most cases, gel droplets are exposed to thermal or UV curing methods which must not harm encapsulated cells. Often, the resulting hydrogel microcapsules may also be re-suspended in a continuous phase of aqueous media so that longer-term cell studies are possible, although some of the benefits of droplet confinement are eliminated in a re-suspended environment [80, 81].

To match the high-throughput droplet generation to downstream droplet processing capabilities, high-throughput actuation and measurement interfaces, data processing and analysis and real-time feedback control are essential. A typical fluorescence sensing system utilizes a fluorescence microscope, fluorescence-inducing light source and a high resolution camera. A laser induced fluorescence system, in combination with a glass-frit nebulizer and a photo-voltaic cell detector, has been described for single molecule detection. The glass-frit nebulizer continuously generates a large number of droplets with an average droplet size of three micrometers in diameter [82]. Single cells are compartmentalized in emulsion droplets, which can be sorted using

dielectrophoresis in a fluorescence-activated manner (as in FACS) at rates up to 2000 droplets/s⁻¹ [83].

However, other types of label-free analysis and detection techniques are also developed. For instance, mass spectrometry (MS) uses a label-free system which ionizes sample compounds to generate charged molecule fragments for microdroplet detection. A MS microchip that integrated multiple modules including a droplet generator, a droplet extraction interface, and a monolithic ESI emitter has been reported. The system performance is evaluated using angiotensin as a model sample and high sensitivity and a good reproducibility are obtained. Raman spectroscopy utilizes non-invasive, label-free interrogation of single cells and droplets using a spectrum of Raman bands of molecular vibration initiated by an incident laser to serve as fingerprint of a cell. An integrated optofluidic Raman-activated cell sorting (RACS) platforms that combine multichannel microfluidic devices and laser tweezers Raman spectroscopy (LTRS) for delivery identification, and simultaneous sorting of individual cells is reported. The system allows label-free cell identification based on Raman spectroscopy and automated continuous cell sorting [84]. A fast, high specific and reliable discrimination of bacteria on strain level by means of Surface-enhanced Raman spectroscopy (SERS) in a microfluidic device is demonstrated. With the applied sample preparation, high specificity and reproducibility of the spectra are achieved [85]. Other studies have used insulated electrodes placed within the top and bottom channel surfaces to measure capacitance as droplets pass between the electrodes, since the droplet changes the overall dielectric constant across the channel. A sensor for the detection of microdroplets in flight, which is based on a capacitive principle, allowing for non-contact monitoring of a complete droplet

dispensing process is reported [86]. Detection of the presence, size and speed of microdroplets in microfluidic devices is presented using commercially available capacitive sensors which make the droplet based microfluidic systems scalable and inexpensive. Cross-contamination between the droplets is eliminated by introducing a passivation layer between the sensing electrodes and droplets [87]. Additional studies include using magnetic nanoparticles to interrogate droplets by nuclear magnetic resonance (NMR) imaging coils wrapped around channels and using suspended droplets and hydrogels as sensors for microdroplet-based living cell analysis [88-90].

2.2.2 Optical detection of living cell in microfluidic devices

For an optofluidic system, optical methods often provide simple and powerful way to control and sense biochemical samples. A biological microbeam has the ability to deliver precise doses of radiation to single cells [91]. The optofluidic manipulation techniques have been considered in biological irradiators, because of the ability to use light beams to remotely handle particles [92].

Optical tweezers have been used to manipulate oocytes in a laser microsurgery device and optical tweezers have been integrated into a synchrotron radiation probe. The study combines microfluidics with optical microablation in a microscopy system that provides high-throughput manipulation of oocytes, automated media exchange, and long-term oocyte observation. The microfluidic component of the system transports oocytes from an inlet port into multiple flow channels. This allows for easy media replacement without disturbing the oocyte location [93]. Another dedicated optical tweezers setup with a compact, portable, and versatile geometry for the customary manipulation of objects for synchrotron radiation applications has been developed. Objects of a few micrometers up to a few tens of micrometers size can be trapped for extended periods of time. The selection and positioning of single objects out of a batch of many can be performed semi-automatically by software routines [94].

On the other hand, an optofluidic platform also allows the usage of a simplified imaging procedures and related applications in biomedicine. The optical imaging of samples in a biological or clinical setting is generally performed with expensive and bulky microscope systems. These can be replaced by of optofluidic microscopes whose major advantages devices are their compactness and low cost while the high resolution

imaging is the most challenging requirement [95]. A miniaturized shadow imaging device for the investigation of the behavioral effects of spaceflight on the nematode *Caenorhabditis elegant*, a widely used model organism for the experimental study of genetics, development, radiobiology, as well as behavior and aging is reported. Severe volume and power constraints for the design of satellite payloads indicate the need to miniaturize of space biology experiments and measurement systems [96]. A novel microfluidics-based lens less imaging technique, termed optofluidic microscopy (OFM) is established, and *Caenorhabditis elegant* imaging with an OFM prototype that gives comparable resolution to a conventional microscope and a quite high measured resolution limit is demonstrated [95].

The most developed optofluidic system for living cell analysis system based on optofluidic device is the flow cytometer, which is a widely used analysis tools in biomedical research and clinical diagnostics. The traditional flow cytometer detects and analyses optical signals (angular light scatter or emitted fluorescence) to identify individual cells or biological samples. After identification by an optical system, a downstream sorting system can isolate cells of interest [97]. An optofluidic based cytometer can provide a compact and low-cost cell sorting capabilities. A single platform flow cytometric absolute CD34+ cell counts is designed and realized. The basic ISHAGE method with the addition of a known number of flow-count fluorospheres is modified to reduce errors inherent to sample washing/centrifugation. The modifications convert the basic protocol into a single-platform method to determine the absolute CD34 count directly from a flow cytometer and form the basis of the Stem-Kit from Coulter/Immunotech [98]. At the same time, significant progress has been made towards

developing microfabricated FACS (μ FACS). A high performance microfabricated FACS system with highly integrated microfluidics, optics, acoustics, and electronics is demonstrated. Single cell manipulation at high speed is made possible by the fast response time of an integrated actuator and a nozzle structure at a sorting junction [99]. To demonstrate point-of-care and home care functions, the device has been applied to measure neutrophil concentration in the blood [100]. Similarly, a unique optofluidic device that can measure optically encoded forward scattering signals is demonstrated. From the spatial pattern, the position and velocity of each cell that are measured in the flow and generate a 2-D cell distribution plot over the cross section of the channel is generated [101]. Furthermore, a compact flow cytometer with liquid-core waveguides and flattened PDMS side surfaces is demonstrated. A single cell event at up to 50 cells with merely a few microliters of sample flows inside the microfluidic. This method can be widely integrated into many applications that combine optoelectronics with microfluidics, especially in bio-sensing, clinical diagnostics, point-of-care testing, and single cell analysis [102].

2.2.3 Bacteriophage Detection and host cell detection

To reduce human health risks from viral infection, drinking water sources have to be constantly monitored to avoid viral contamination [103]. Bacteriophages are viruses that infect bacterial cells. The concentration of bacteriophages in water sample is significantly correlated with other type virus. Therefore, bacteriophages have long been considered as attractive indicators for determining drinking water quality [21, 104]. Furthermore, the detection of bacteriophages is also important since the infection of bacterial cultures by bacteriophages may lead to loss of a desired product and spreading of bacteriophages throughout an entire laboratory [105].

There have been two principal methods of growing a virulent phage, both based on the ability of the phage to kill the host bacterial cell. One method introduces phage into a fluid bacterial culture medium. After a period of incubation, the phages lyse the bacteria in the broth culture resulting in a clearing of the fluid medium. The other method, known as the plaque assay, introduces phages into a few milliliters of soft agar along with some bacterial host cells. After a period of incubation, the phages lyse the bacterial cells in their vicinity resulting in zones of clearing on the plate known as plaques. Current bacteriophage detection techniques are mainly adapted from the clinical applications based on the plaque assay method using host cells and polymerase chain reaction (PCR) methods [106]. Limitations of these techniques include long processing time for the culture assay and a complicated pre-concentration step before target detection can be achieved. Researchers also developed bacteriophage detection methods using special electric DNA chip or surface modified quartz crystal microbalances, which have higher efficiency and sensitivity. The principle of the electric bio-chips is to capture the target

molecules (either nucleic acids or proteins) on the chip, and the use of the a secondary detection probe which is coupled with an enzyme catalyzing a red-ox reaction [107]. It is also possible to detect specifically adsorbed bacteriophage directly by breaking the interactions between proteins displayed on the phage coat and ligands immobilized on the surface of a quartz crystal microbalance (QCM) [108]. However, these techniques required complex fabrication process and the detection device can just detect only a specific target bacteriophage. It is difficult to realize rapid, sensitive and low-cost monitoring and detection low concentration of bacteriophages in water samples.

Previously, important steps have been made to perform droplet-based manipulations of cells such as cell encapsulation, on-chip incubation and cell screening [53, 109]. The fluorescent based cell quantification and detection is used most frequently. The physical and chemical isolation of droplets eliminates the risk of cross-contamination of different dye while fast and efficient mixing of the reagents can take place occurring inside the droplets [110]. However, because of the isolated microenvironment of the droplet carrier, the cell as bacteria need to be pre-stained with the fluorescent dye before droplet formation and this may also influence cell growth for long-term incubation. Moreover, the dye is very difficult to wash during the detection process, which causes inaccuracy of quantification [111, 112].

In contrast, the optofluidics techniques further extend the functionalities of a microfluidic system by integrating optical systems into it. For instance, the dry/wet mass of a single living cell has been measured using an optofluidic chip using interferometry to determine a single living cell's dry/water mass using optofluidic chip [113]. Furthermore, by observing the optical scattering pattern of single microdroplet containing different

amount of host cells, no more fluorescent dye or other indicators are necessary, which simplified the set-up on the pre-treatment protocol and also enables a real-time, label-free detection [114]. The optical signal can then be convenient to detect and can processed by signal analysis circuits. Some previous studies developed methods to evaluate the concentration of include particles with known size and refractive index in droplet carriers by light-scattering simulations [115, 116]. Most of the previous analyses are too theoretical to real host cell study. As the same time, the optical set up they used is relied on an open system, which is not suitable for long time host cell incubation and observation stability.

2.3 Endothelial cell and diabetes

2.3.1 Diabetes and hyperglycemia influence to endothelial cell

Diabetes mellitus is a disease with high morbidity and mortality worldwide, which is a serious threat to human health. The deficiency of blood glucose control in patients of Type 2 diabetes increases the risk of microvascular end-points associated with peripheral neuropathy, nephropathy and retinopathy as well as macrovascular diseases such as atherosclerosis [25, 117, 118].

A wide variety of studies suggest that hyperglycemia is the primary cause of the development and progression of abnormal vascular growth and the complications of diabetes, whose major risk factor is the dysfunction of endothelial cells under high glucose concentration. In diabetes mellitus, hyperglycemia is usually caused by low insulin levels (Diabetes mellitus Type 1) and/or by resistance to insulin at the cellular level (Diabetes mellitus Type 2), depending on the type and state of the disease. Low insulin levels and/or insulin resistance prevent the body from converting glucose into glycogen (a starch-like source of energy stored mostly in the liver), which in turn makes it difficult or impossible to remove excess glucose from the blood [119-121]. With normal glucose levels, the total amount of glucose in the blood at any given moment is only enough to provide energy to the body for 20-30 minutes, and so glucose levels must be precisely maintained by the body's internal control mechanisms. When the mechanisms fail in a way that allows glucose to rise to abnormal levels, hyperglycemia is the result [122-124].

Previously, others have demonstrated the underlying molecular mechanism of how hyperglycemia causes endothelial cell apoptosis and necrosis such as polyol

pathway flux [120], oxidative stress [119], non-enzymatic glycation and the diacylglycerol–protein kinase C (DAG–PKC) activation [122]. Among them, the main reason is the overproduction of reactive oxygen species (ROS) caused by oxidative stress, which is related to PKC-dependent NAD(P)H oxidase activation and the down-regulation of activity and the expression of enzyme Endothelial Nitric Oxide Synthase (eNOS) [123, 124].

Reactive oxygen species (ROS) are natural byproducts of the normal aerobic metabolism in cells, which include superoxide anion ($O_2^{\bullet-}$), hydroxyl radicals ($OH^{\bullet}$), hydrogen peroxide molecules (H_2O_2), etc [125]. The main source of ROS production is the mitochondrion during oxidative phosphorylation, which involves the electron transport chain [126]. In the chain, electrons are passed through several proteins by different oxidation-reduction reactions. However, a small percentage of the electrons are leaked out from the mitochondrion electron transport chain, and subsequently superoxide anions are generated. Other cellular sources of ROS production include enzymatic reactions, signal transduction, antimicrobial action of white blood cells, etc. [127]. For example, xanthine oxidase catalyzes the oxidation of hypoxanthine to xanthine and further to uric acid by generating superoxide anions. Similarly, reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which is a membrane-bound enzyme complex generates superoxide anions by transferring electrons from NADPH inside the cell across the membrane and couples the electrons to the oxygen molecules. Superoxide anions have limited reactivity by itself, but they can directly inactivate specific enzymes such as the reduced NADPH complex of the mitochondrial electron transport chain. To maintain the superoxide anion at the basal level and achieve homeostasis, the cell has

antioxidant defense system to convert superoxide anions to hydrogen peroxide. For example, high concentration of manganese superoxide dismutase (MnSOD) in the mitochondrial matrix catalyzes the breakdown of superoxide anion to hydrogen peroxide and avoids the damage of proteins involved for the regulation of metabolism.

Hydrogen peroxide is a poor reactive oxidant agent but it is cell membrane-permeable. Under normal metabolic condition, hydrogen peroxide is involved in the regulation of the signal transduction events [128]. For example, hydrogen peroxide inhibits crucial phosphatases that attenuate signal propagation from activated growth-factor receptors. Hydrogen peroxide is also generated by several enzymatic systems inside the cells, such as phagocytic oxidases in the plasma membrane, sulphhydryl oxidase in the endoplasmic reticulum, lipid oxygenase in cytosol, etc. This shows that the intracellular concentration of hydrogen peroxide is tightly regulated. However, similar to superoxide anions, excess hydrogen peroxide will cause cellular toxicity. Hydrogen peroxide can react with ferrous iron to form hydroxyl radicals, which are short-lived ROS and can react with DNA molecules, membrane lipids, and carbohydrates. Excess hydrogen peroxide is broken down by catalase and glutathione (GSH/Px) to yield water molecules.

In overall, the antioxidant defense system of cell ensures the ROS level is kept at the basal and nontoxic level during normal aerobic metabolism. This includes also non-enzymatic systems such as glutathione, ascorbate, vitamin, etc. However, oxidative stress is induced when the excessive ROS production level overcomes the antioxidant defense system of the cell and causes the loss of homeostasis. Oxidative stress can lead to cell damage, apoptosis, and modifications to cellular proteins, lipids, and DNA. The level of

oxidative stress on cell can be determined by monitoring the morphological change of the mitochondria since mitochondria are part of the pathway for cell apoptosis [129]. Normally, mitochondria in endothelial cells form a filamentous reticular network and constantly undergo morphological change through fusion and fission [130, 131]. During apoptosis, mitochondria are fragmented via fission, and individual mitochondrion becomes swollen and loses its membrane potential.

There are several reasons for the excess levels of ROS production in cells. First, short term elevation of ROS level occurs after exhaustive exercise [132]. Second, the presence of exogenous ROS sources can also elevated ROS level, such as ultraviolet light, ionizing radiation, inflammatory cytokines, environmental toxins, etc. Third, diseases can also increase the ROS level, such as hypertension [133], renal failure, diabetes [134, 135] cardiac disease, etc. Therefore, researchers have put in efforts to investigate the relationship between the elevated ROS level, the ROS-related diseases and potential drugs with antioxidant ability [136]. For example, high glucose level [137] in diabetes patients and high sodium level [138] in renal failure patients have been proven to increase the ROS production level in cells. In addition, different antioxidants or ROS scavengers are studied and shown to reduce ROS production level. However, these studies are conducted under either a static condition in culture dishes or under a constant shear stress [139], which are different from the physiological condition with pulsatile shear stress.

2.3.2 Endothelial cell response to physical stimulations

However, another important factor, shear stress, which is imposed tangentially to the cell surface caused by the pulsatile hemodynamic forces in the blood vessels, has often been ignored.

The hemodynamic conditions inside blood vessels lead to the development of superficial stresses near the vessel walls, which can be divided into two categories: circumferential stress due to pulse pressure variation inside the vessel and shear stress due to blood flow [139]. The direction of the shear stress vector is determined by the direction of the blood flow velocity vector very close to the vessel wall. Shear rate is defined as the rate at which adjacent layers of fluid move with respect to each other, usually expressed as reciprocal seconds [140, 141]. Appropriate pulsatile shear stress is a well-known critical factor in the development of blood vessel structure either during early growth or for adults. The vascular wall is constantly subjected to physical forces, which regulate important physiological blood vessel responses, as well as being implicated in the development of arterial wall pathologies. Changes in blood flow, thus generating altered hemodynamic forces are responsible for acute vessel tone regulation, the development of blood vessel structure during embryogenesis and early growth, as well as chronic remodelling and generation of adult blood vessels [142, 143]. On the other hand, some researchers have found that exposure to high level shear stress caused oxidative stress on the endothelial cells and further induced cell apoptosis or necrosis. In the presence of hyperlipidaemia, disturbed blood flow results in increased endothelial turnover in the arterial wall. It is demonstrated that disturbed blood flow activates

endoplasmic reticulum stress initiating a signal pathway leading to endothelial apoptosis [139, 144, 145].

In vitro models have been used previously to investigate shear stresses applied to endothelial cells under various flow conditions: 1) Steady, laminar flow using parallel planar or conical moving surfaces. An *in-vitro* system for studying the dynamic response of vascular endothelial cells to controlled levels of fluid shear stress is developed. Cultured monolayers of bovine aortic endothelial cells are placed in a cone-plate apparatus that produces a uniform fluid shear stress on replicate samples. Confluent monolayers undergo a time-dependent change in cell shape from polygonal to ellipsoidal and become uniformly [146]. 2) Unsteady, pulsatile, laminar flow by using peristaltic pumps which allow the study of time varying shear stresses. *In vivo* endothelial cells are exposed to different types of pulsatile stress conditions. The results show that in a type I nonreversing flow, cell shape changed less rapidly, but cells took on a more elongated shape than their steady flow controls long-term [147]. 3) Oscillatory, turbulent flow. The oscillating shear stress renders the endothelial cells less responsive to simvastatin than cells exposed to unidirectional steady or pulsatile flow. Consequently, the pleiotropic effects of statins *in vivo* may be less effective in endothelial cells exposed to atheroprone hemodynamic [148]. Advanced experimental techniques also exist that allow the 3-D analysis of shear stresses and deformation of endothelial cells [149, 150].

However those studies are performed under laminar shear stress with either constant or variable flow rate that are different from the real physiological conditions in the blood vessels with a pulsatile shear stress. The pulsatile studies require open systems, in which is difficult to prevent influences of any external factors and contamination. Thus,

it is necessary to introduce the pulsatile shear stress into the traditional model to reflect the fluctuating flow rate of the blood stream in the human body. Moreover, the combinational effect of the two factors: hyperglycaemia and shear stress, which compose the microenvironment of endothelial cell growth on endothelial cells, is still not clear.

2.3.3 Endothelial cell dysfunction and death detection

The process of programmed cell death, or apoptosis, is generally characterized by distinct morphological characteristics and energy-dependent biochemical mechanisms. Apoptosis is considered a vital component of various processes including normal cell turnover, proper development and functioning of the immune system, hormone-dependent atrophy, embryonic development and chemical-induced cell death [151]. Apoptosis occurs normally during development and aging and as a homeostatic mechanism to maintain cell populations in tissues. Apoptosis also occurs as a defense mechanism such as in immune reactions or when cells are damaged by disease or noxious agents [152].

Apoptosis can be detected by a number of standard assays including the agarose gel analysis for detecting DNA fragmentation, the TUNEL (Terminal dUTP Nick End-Labeling) assay for detecting DNA damage, the caspase activity assay, The western blot analysis for detecting the cleaved form of PARP and DNA staining for revealing chromatin condensation, etc. [151, 153, 154]. Light and electron microscopy can identify various morphological changes that occur during apoptosis [155]. Light microscopy can observe cell shrinkage and pyknosis, which is the result of chromatin condensation, during the early process of apoptosis [156]. Electron microscopy can better define the subcellular changes. Early during the chromatin condensation phase, the electron-dense nuclear material characteristically aggregates peripherally under the nuclear membrane [157]. The TUNEL method is used to assay the endonuclease cleavage products by enzymatically end-labeling the DNA strand breaks [158]. However, most of these

methods require a large number of cells for analysis and complicated experimental procedures. More importantly, they cannot be used to detect apoptosis in live cells.

Fluorescence-based DNA staining has been used to detect DNA fragmentation and chromatin condensation for indicating apoptosis in live cells. However, this method is usually used as an end point assay as prolonged incubation of the dye molecules with cells may cause DNA damage which itself may cause cell death. Fluorescence resonance energy transfer (FRET) is a mechanism describing energy transfer between two chromophores. A donor chromophore, initially in its electronic excited state, may transfer energy to an acceptor chromophore through nonradiative dipole–dipole coupling. The efficiency of this energy transfer is inversely proportional to the sixth power of the distance between donor and acceptor making FRET extremely sensitive to small distances [159-161]. One example is the FRET sensor developed from spectrin-repeat stress-sensitive FRET (sstFRET), which matched the mechanical compliance of common hosts by substituting a spectrin repeat for the linker [162] to a Time Series Modelling (TSMoD) FRET sensor using a domain from spider silk as the linker [163], finally as a train sensor based on proximity imaging (PRIM) with green fluorescent protein (GFP) dimers [164]. These force sensors share a uniform mechanism for interpreting force: tension in the host induces strain in the linker, leading to increased distance between the donor and acceptor. The dynamic range of these sensors is limited by the nearly linear relationship between FRET efficiency and strain [165, 166].

Another example is caspase sensor based on Förster resonance energy transfer between fluorescent proteins. An enhanced cyan fluorescent protein anchored to the inner leaflet of the plasma membrane of living cells is optically excited by an evanescent

electromagnetic field and transfers its excitation energy via a spacer (DEVD) to an enhanced yellow fluorescent protein [167]. Specific caspase sensors preferentially cleaved by caspase-3, -6 or -9 are expressed in differentiated human neuroblastoma SH-SY5Y cells. The anchoring of the sensors resulted in high FRET signals both in extended neuritis and soma and made analysis of spatio-temporal signal propagation possible [168].

2.4 Summary

Based on the literature survey presented, optofluidic techniques provide various possibilities to replace traditional optical devices and have developed the better optical performance and manipulation flexibility. The microdroplet techniques have been shown to be powerful tools in the study of different areas. The combination of optical detection with droplet-based microfluidic devices plays a vital role in the development of optofluidic devices. For an optofluidic system, the optical methods often provide simple and powerful ways for sensing and characterizing biochemical samples. The microdroplet techniques, on the other hand, provide various methods to manipulate and control the sample. It is possible to develop an integrated optical system based on the microdroplet techniques for cell sorting, encapsulation, trapping, measurement and characterization.

Hyperglycemia is the primary cause of the development and progression of abnormal vascular growth and the complications of diabetes and, therefore, its effect has to be studied on live cells in such optofluidic chips. Moreover, it is necessary to introduce the pulsatile shear stress into the model to reflect the fluctuating flow rate of the blood stream in the human body. An optofluidic hemodynamic system makes it is possible to study on living cells. The combination effect of the two factors, hyperglycemia and shear stress, which compose the cell growth microenvironment of endothelial cells.

CHAPTER 3

DROPLET OPTOFLUIDIC GRATING

This chapter presents the design, fabrication and testing of a droplet optofluidic grating. The principle of microdroplet generation and manipulation are described. An equation which describes the relationship between the droplet size and flow rates of two immiscible phases is deduced. The design of a reconfigurable optical diffraction grating using multiphase droplets is demonstrated and the optical properties of the diffraction pattern of the droplet grating are analyzed. Subsequently, the fabrication processes of the optofluidic chip based on soft-lithography using PDMS, and the packaging and integrating of the microfluidic system is illustrated. Finally, the optical performance of the droplet optofluidic grating is determined experimentally. It is demonstrated that it exhibits high stability and tunability in grating period and refractive index variation, enabling to obtain various diffraction patterns and thus having high potential applications. A phase-transmission-grating-based compact optofluidic refractometer and a zero-order transmission color filter have been realized based on the diffraction pattern analysis.

3.1 Design of the droplet optofluidic grating

3.1.1 Multiphase microdroplets generation

Fluid is defined as a continuous, amorphous substance whose molecules move freely past one another and which has the tendency to assume the shape of its container. Microfluidic is defined as the study and application of fluid flow on the microscale. Phase is a distinct state of aggregation of matter, matter that is identical in chemical composition and physical state and separated from other material by the phase boundary. Multiphase fluids are those in which there exist at least two different fluids in a system with different chemical compositions. Compared to a single-phase flow, the corresponding multiphase microfluidic flow is more complicated even in a simple configuration.

A wide variety of physical phenomena occur in microfluidic devices, the importance of which must be judged against competing phenomena. Dimensionless numbers expressing the ratio of these phenomena give a sense for where a system sits in fluidic parameter space. Of all dimensionless numbers, the Reynolds number (Re) is one most often mentioned in connection with microfluidics, which presents the relating importance of inertial forces to viscous forces that is expressed as

$$\text{Re} = \frac{\rho u L}{\mu} \quad (2.1)$$

where ρ is the density, u is the average flow velocity, μ is the viscosity of the fluid respectively, L is the characteristic flow dimension. Almost without exception, microfluidic devices are characterized by small Reynolds numbers, measuring that the

small enough for that viscous force typically overwhelms inertial forces, and the resulting flows are laminar and predictable [169].

Flow in microchannels can be driven not only by external pressure or forces generated by an electrical, thermal, photonic or phonic field, but also by internal forces which are determined by fluid and channel properties such as interfacial forces. Physically, these forces acting on fluids can be split into normal and tangential components. In multiphase microchannel flows, the viscous forces mainly act tangential to the liquid-gas interface elongating it, whereas the interfacial forces mainly act normal to the interface inducing droplet formation. The capillary number is hereby used to express the competition among these two types of forces as [170]

$$Ca = \frac{\mu u}{\sigma} \quad (2.2)$$

where μ is the viscosity, u is the mean speed of the carrier fluid, σ is the interfacial tension. At low values of capillary number, when the interfacial forces dominate the shear stresses, the dynamics of break-up of immiscible threads in T-junctions is dominated by the pressure drop across the droplet as it forms. The shear stresses exerted on the interface of the emerging droplet are not sufficient to distort it significantly. Consequently, the droplet blocks almost the entire cross-section of the main channel and confines the flow of the carrier fluid to thin wetting films on the walls of the microchannel. This leads to an increase of pressure upstream of the emerging droplet and leads to “squeezing” of the neck of the immiscible thread.

Another important characteristic of a fluid is its contact angle, which becomes evident when a liquid droplet contacts both a solid and a gas (or a solid and a second

Figure 3.1: Wettability of aqueous droplet: (a) On free surfaces and (b) In microchannels.

immiscible liquid), as shown in Fig. 3.1. The liquid curvature is caused by the differences among interfacial tensions. Young's equation for the force balance can be expressed as

$$\sigma_{sg} = \sigma_{sl} + \sigma_{lg} \cos \theta \quad (2.3)$$

where σ indicates the interfacial tension between two phases, the subscripts g , l , s represent gas, liquid and solid, respectively, and θ is the contact angle. Therefore, in a hydrophobic channel, concave and convex interfaces are created upon filling in, depending on whether the channel walls are hydrophobic or hydrophilic, respectively, as shown in Fig. 3.1.

A typical process of formation of droplets in the T-junction can be described as follows: the two immiscible fluids form an interface at the junction of the inlet and main channel; the stream of the discontinuous phase penetrates into the main channel and a droplet begins to grow; the pressure gradient and the flow in the main channel distort the droplets in the downstream direction; the interface on the upstream side of the droplet moves downstream; when the interface approaches the downstream edge of the inlet for

Figure 3.2: Schematic of a T-junction microchannel for droplet generation.

the discontinuous phase, the neck connecting the inlet channel with the droplet breaks; the disconnected liquid plug flows downstream in the main channel, while the tip of the stream of the discontinuous phase retracts to the end of the inlet and the process repeats [49].

The geometry of the T-junction is illustrated in Fig. 3.2. Two channels merge at the right angle. The main channel carries the continuous fluid and the orthogonal channel supplies the fluid that will be dispersed. The channels have rectangular cross-sections, and the geometrical parameters that define the size and the shape of the T-junctions are: the width of the main channel w , and the height of the channel h , while L is defined as the length of the immiscible slug; $Q_{aqueous}$ and Q_{oil} is the flow rates of the dispersed and continuous fluids, respectively.

The size of the droplet can be quantitatively predicted. First, the tip of the discontinuous phase enters and blocks the main channel. At this moment, the length of

the droplet is approximately equal to the width of the channel. The increased pressure in the continuous fluid upstream of the immiscible thread starts to squeeze the neck (of characteristic width d). The thickness of the neck decreases at a rate which is approximately equal to the mean speed of the flow of the continuous fluid: $u_{squeeze} \approx u = \frac{Q_{oil}}{hw}$. During this process the droplet elongates at rate $u_{growth} \approx \frac{Q_{aqueous}}{hw}$. The final length

of the droplet is therefore equal to: $L \approx w + \left(\frac{d}{u_{squeeze}} \right) u_{growth} = d \frac{Q_{aqueous}}{Q_{oil}}$. It is convenient to

non-dimensionalize can be given by

$$\frac{L}{w} = 1 + \alpha \frac{Q_{aqueous}}{Q_{oil}} \quad (2.4)$$

where L is the length of the immiscible slug, w is the width of the channel, $\frac{Q_{aqueous}}{Q_{oil}}$ are the flow rates of the dispersed and carrier fluids respectively, and α is a constant equal to d/w , whose particular value depends on the geometry of the T-junction. The scaling relation is independent of the material parameters of the fluid their viscosities and the interfacial tension between them [45].

The refractive index of the microdroplet can also be well controlled. It is frequently pointed out in light-scattering and other studies that the refractive index of a solution increases linearly with concentration, when the concentration is expressed in terms of weight of solute per unit volume of solution, that is, $\frac{w}{v}$ units. However, in many analytical applications of refractive index measurements, concentration is normally

expressed in terms of weight of solute per unit weight of solution, that is, $\frac{w}{w}$ units, and

conversion into $\frac{w}{v}$ units requires knowledge of the density of each sample. “Table 3.1

presents the variation with concentration for the refractive index of CaCl_2 solution with different concentration. The refractive index of the CaCl_2 solution is measured using refractometer.” By using such solutions as the dispersed phase to form microdroplets, the refractive index of the microdroplets is easily controlled.

Table 3.1 Relationship between concentration and refractive index of calcium chloride solution

Calcium chloride concentration vs. refractive index (20 °C)	
% by wt.	n_D
0.00	1.3330
2.00	1.3378
4.00	1.3426
6.00	1.3475
8.00	1.3525
10.00	1.3575
12.00	1.3625
14.00	1.3677
16.00	1.3730
18.00	1.3784
20.00	1.3839
24.00	1.3951
28.00	1.4066
32.00	1.4183
36.00	1.4301
40.00	1.4420

3.1.2 Diffraction pattern analysis

Diffraction gratings are usually divided according to several criteria. Intuitively it is much easier to distinguish between reflection and transmission gratings. They can also be categorized as phase gratings and relief gratings. Phase gratings are considered to consist of refractive index variations while relief gratings consist of a surface relief structure that separates two media of different optical properties as shown in Fig. 3.3 (a-b).

Figure 3.3: Types of diffraction gratings: (a) Phase grating (b) Relief grating and (c) Generation of diffraction pattern orders after transmission through an array of small slits.

Usually reflection gratings are relief gratings, covered with some highly reflecting material. Depending on the spectral region reflectivity can vary significantly, so that the choice of material can be critical for grating performance. Although the majority of spectrometric instruments are designed around reflection gratings, there are a number of situations where transmission gratings are preferred. Transmission gratings are usually made with triangular, sinusoidal or rectangular groove shapes. Triangular or blazed grooves are designed to direct as much light as possible of a specified wavelength band into one of the low orders, usually the first, or to deliver a particular ratio of first to zero order at a given wavelength as shown in Fig. 3.3 (c). At normal incidence the symmetry of rectangular grooves leads to equal energy in both plus and minus first orders, while the fraction devoted to the zero order at one wavelength can vary from near 0 to over 90 %. It is generally designated as phase gratings, because their behavior is controlled by the physical phase retardation between light originating from successive grooves.

The liquid droplet array grating consists of two regions with different refractive indices as shown in Fig.3.4: the immersion oil with refractive index n_{oil} and droplets with refractive index $n_{droplet}$. With constant refractive indices of the two immiscible liquid, the diffraction pattern of the droplet array is a combination of single circular aperture Fraunhofer diffraction and a multiple-slit interference as shown in Figs. 3.5(a)-(b). The circular diffraction pattern is determined by the single droplet diameter. The irradiance distribution complies with the diffraction equation of a single circular aperture, which can be expressed as

$$I(\theta) = I_0 \left(\frac{2J_1(ka \sin \theta)}{ka \sin \theta} \right)^2 \quad (3.5)$$

where a is the diameter of a single droplet. The period (i.e. radius of the 1st, 2nd and 3rd maxima) is inversely proportional to the single droplet diameter:

$$y_1 = \frac{0.81\lambda}{as} \propto \frac{1}{a} \quad (3.6a)$$

$$y_2 = \frac{1.33\lambda}{as} \propto \frac{1}{a} \quad (3.6b)$$

$$\text{nd} \quad y_3 = \frac{1.85\lambda}{as} \propto \frac{1}{a} \quad (3.6c)$$

The vertical pattern is determined by the droplet array dispersed in immersion oil which induces periodic alterations in refractive index, i.e. the droplet array acts as a phase grating. For any type of grating, the foundational grating equation holds:

$$d \sin \theta_m = m\lambda \quad (3.7)$$

where d is the grating period and θ_m is the diffraction angle of the order m . The period of the vertical diffraction pattern (i.e. the displacement between two consecutive maxima) is inversely proportional to the length of grating period:

$$\Delta y \approx s(\theta_m - \theta_{m-1}) = \frac{s\lambda}{d} \propto \frac{1}{d} \quad (3.8)$$

If the incident beam is multi-wavelength instead of single-wavelength, the droplet-based transmission grating also shows dispersion effect. For a fixed direction of incidence, the direction of each principal maximum varies with wavelength. Figs. 3.5(c)-(d) shows the angular spread of the continuous spectrum of visible light for the grating which is given by the grating equation 3.7, from which it results that

$$\theta_m = \sin^{-1} \frac{m\lambda}{d} \quad (3.9)$$

Overlapping of different orders may occur because the product may be equal to several possible combinations of m and λ of the incident light. The amount of overlap also depends on the grating density, which is determined by the grating period d as shown in Fig. 3.6.

Figure 3.4: (a) Schematic of the droplet optofluidic grating; (b) Top view of the droplet optofluidic grating and (c) Cross view of the droplet optofluidic grating.

Figure 3.5: Diffraction pattern analysis of the droplet grating (a-b) Circular diffraction pattern of single droplet and (c-d) Vertical diffraction pattern of droplet array.

Figure 3.6: Angular spread of the first three orders of the spectrum for the droplet optofluidic diffraction grating.

3.1.3 Phase transmission optical properties

The droplet-based phase transmission grating consists of alternated regions of immersion oil and droplets with different refractive indices, n_{oil} and $n_{droplet}$. The periodic phase shift ξ is given by

$$\xi = \frac{2\pi h(n_{oil} - n_{droplet})}{\lambda} \quad (3.10)$$

where h is the height of the microfluidic channel (i.e., the thickness of the grating structure), λ is the wavelength of the incident beam, and $(n_{oil} - n_{droplet})$ is the refractive index contrast of two liquids. The droplet profile also affects the phase shift as depicted in Fig. 3.7. When the contact angle is 90°, the droplet has an ideal profile of rectangular cross section as shown in Fig. 3.7(a). With the reduction of the contact angle, the droplet profile is rounded and thus the wavefront phase shift profile is distorted as shown in Fig. 3.7(b-c). This kind of distortion would affect the diffraction patterns. For small droplets, the profile could be in a nearly circular shape. This study focuses particularly on the ideal condition, though the design would work equally for the other profiles [171].

Suppose that the total length of the grating is L , the transmission function of a phase grating $t(x)$ can be considered as the addition of two functions, $t_1(x)$ and $t_2(x)$, which are expressed as

$$t(x) = [t_1(x) + t_2(x)] \text{rect}\left(\frac{x}{L}\right) \quad (3.11)$$

Figure 3.7: The wave front phase shift profiles for different types of droplets.

where

$$t_1(x) = \frac{1}{d} \text{rect}\left(\frac{x}{a}\right) \text{comb}\left(\frac{x}{d}\right) \exp\left(\frac{j2\pi h n_{\text{droplet}}}{\lambda}\right) \quad (3.12a)$$

and

$$t_2(x) = \frac{1}{d} \text{rect}\left(\frac{x-d/2}{b}\right) \text{comb}\left(\frac{x}{d}\right) \exp\left(\frac{j2\pi h n_{\text{oil}}}{\lambda}\right) \quad (3.12b)$$

It is known that the field distribution of Fraunhofer diffraction of an aperture is the Fourier transform of the amplitude distribution across the aperture. As the plane wave is normally incident on the grating, the amplitude distribution across the grating can be described by the transmission function $t(x)$ of the grating. For convenience, suppose that $a = b = d/2$, making Fourier transformation of Eq. 3.11 will have

$$T(f_x) = 2a \left\{ \sum_{n=-\infty}^{+\infty} \sin c\left(\frac{m}{2}\right) \delta(df_x - m) \times \exp\left[j\pi\left(\frac{h(n_{\text{droplet}} + n_{\text{oil}})}{\lambda} - \frac{m}{2}\right)\right] \right\} L \sin c(Lf_x) \quad (3.13)$$

Therefore, the intensity distribution of the Fraunhofer diffraction of the phase grating can be expressed as

$$I(f_x) = L^2 \sum_{n=-\infty}^{+\infty} \sin^2 \left(\frac{m}{2} \right) \cos^2 \left(\frac{\xi}{2} + \frac{m\pi}{2} \right) \times \sin^2 \left[L \left(f_x - \frac{m}{d} \right) \right] \quad (3.14)$$

when $m = 0$, Eq. 3

$$I_0(f_x) = L^2 \cos^2 \left(\frac{\xi}{2} \right) \times \sin^2 (L f_x) \quad (3.15)$$

This equation represents the intensity distribution of the zero diffraction order.

Then the peak intensity of the zero diffraction order is

$$I_{0\text{peak}} = L^2 \cos^2 \left(\frac{\xi}{2} \right) \quad (3.16)$$

Similarly, if $m = 1$, we have

$$I_1(f_x) = L^2 \frac{4}{\pi^2} \sin^2 \left(\frac{\xi}{2} \right) \times \sin^2 \left[L \left(f_x - \frac{1}{d} \right) \right] \quad (3.17)$$

$$I_{1\text{peak}} = L^2 \frac{4}{\pi^4} \sin^2 \left(\frac{\xi}{2} \right) \quad (3.18)$$

Since L is a constant, Eq. 3.17 and 3.18 can be finally simplified as

$$I_{0\text{peak}} = \cos^2 \left(\frac{\xi}{2} \right) \quad (3.19a)$$

and

$$I_{1\text{peak}} = \frac{4}{\pi^4} \sin^2 \left(\frac{\xi}{2} \right) \quad (3.19b)$$

In the same way, the peak intensities of the higher diffraction orders can also be derived by making $m = 2, 3, \dots, n$. We can see that the most diffraction energy concentrate in the zero order and the first order. It is found that, the value of the peak intensities of both zero order and first order vary with the change of grating thickness h , the refractive indices difference between two immiscible liquids $n_{oil} - n_{droplet}$ and the

Figure 3.8: Simulation results of the diffracted efficiency of the zero-order and first-order diffraction as a function of droplet refractive index.

incident wavelength λ . For monochromatic incident light, when the phase shift ξ changes, the diffracted efficiency of the zero order and first order oscillated periodically, as shown in Fig. 3.8. For broadband visible incident light, this means that different wavelength will be transmitted at different efficiencies, and the wavelength that is diffracted will be filtered out from the 0th order. As all wavelength components of the 0th order are overlapped in the same position, the filtering-out of one or more wavelengths component will result in a subtractive color modification of the output spectrum as shown in Fig. 3.9

Figure 3.9: Simulation results of the spectrum shift of the zero-order with four different droplet refractive indices.

3.2 Fabrication and packaging

3.2.1 Fabrication processes

Polymers are the most promising materials for the fabrication process of the biochips because they are biocompatible, inexpensive, easy to use and applicable for mass replication technologies. Various polymer materials are currently used for microfabrication processes, which include polydimethylsiloxane (PDMS), polymethyl methacrylate (PMMA), polystyrene (PS) and polycarbonate (PC). Polymer materials provide excellent transparency, low density, high heat deflection temperatures and high dimensional stability. The fabrication process for these polymers is known as soft lithography or molding technology, such as injection molding, hot embossing and casting.

PDMS is commercially available in the market (Dow Corning, Gelest) and widely used in microfluidic devices. It is optically transparent for wavelengths down to 300 nm, electrically insulating, chemically inert, odorless and biocompatible. Some of the chemical and physical properties of PDMS are listed in Table 3.2.

The fabrication process for PDMS devices shown in Fig 3.10 involves three main steps, namely master patterning, molding and bonding. Firstly, the master is fabricated on the silicon wafer using lithography techniques, such as SU-8 development. Then, the PDMS elastomer is poured onto the master and thermally cured to obtain the replica. Finally, the replica is peeled off from the master and bonded with PDMS or glass slide to form biochips with sealed microchannels.

Figure 3.10: Fabrication process flow of PDMS microfluidic devices

Table 3.2 The chemical and physical properties of PDMS [172]

Formula	$[\text{Si}(\text{CH}_3)_2\text{O}]_n$
Density (kg/m^3)	965
Chemical resistance	Organic solvent – Cause swelling Alcohol & polar solvent - Excellent
Tensile strength (MPa)	6.2
Glass transition temperature, T_g ($^{\circ}\text{C}$)	120
Dielectric constant	2.65 – 2.7
Water absorption	0.1 %
Refractive index	1.41 – 1.43

3.2.2 Fabrication and packaging

For SU-8 development to do the master patterning (negative tone PR), a layer of SU-8 (SU8 2025) is spin-coated on the silicon substrates, at 500 rpm for 5 s and then immediately ramped to 200 rpm for 30 s to obtain a thickness of 50 μm . Next, the wafer is kept in horizontal state for two hours to flatten and smooth the SU-8 layer. Afterwards, the wafer is put on a hotplate for soft-baking at 65 $^{\circ}\text{C}$ for 15 min and at 95 $^{\circ}\text{C}$ for 30 mins. The wafer is then undergone UV exposure by a mask aligner machine at 24 mW/cm^2 for 8 s. The exposed wafer is then baked again at 65 $^{\circ}\text{C}$ for 1 min and 95 $^{\circ}\text{C}$ for 3 mins. The post exposure bake (PEB) is to catalyze the cross-linking process of the exposed SU-8. Finally, the wafer is immersed into the SU-8 developer for 6 mins. The developed SU-8 structures are attached on the wafer and the master is ready. The recipe for the SU-8 process is provided by the manufacturer and optimized during the fabrication process.

PDMS elastomer (Sylgard 184) is a two-component heat curing system, i.e. it consists of a base part and a curing agent part and they are mixed at the ratio of 10:1. Both parts are poured onto a beaker and stirred thoroughly. The mixture is then degassed in desiccators under low vacuum for 1 hour. After that, the mixture is simply poured onto the master to a certain height and cured for 1 hour at 75 $^{\circ}\text{C}$. The curing temperature and the amount of the curing agent determine the stiffness and optical properties of the PDMS. Moreover, at higher temperature, the PDMS will shrink more. Therefore, a lower temperature and longer baking time are required. After curing, the PDMS sheet can be peeled off from the master. To avoid the stick between the dichloromethylsilane (PDMS) and the master, the master surface has to be treated first. The master is immersed in the

Figure 3.11: Micrograph of the PDMS microfluidic chip.

toluene solvent for 5 hours for the surface treatment process. Several drops of PDMS are tipped into the solvent to enhance the treatment effect.

For the bonding of the PDMS biochip, the PDMS slab and the cover, either a glass slide or another sheet of PDMS polymer, are carefully cleaned with ultrasonic cleaner and isopropanol (IPA) and dried under a gentle stream of nitrogen. After cleaning, both surfaces have to be plasma treated with a handheld corona treater. Cured PDMS has a very low surface energy and is hydrophobic. It is difficult for chemical species to interact with the PDMS surface and the adhesion is generally very poor. Changing the surface characteristics of the PDMS to achieve a higher surface energy will enhance the adhesion characteristics of the PDMS. Therefore, the PDMS surface can be oxidized by plasma treatment. The corona discharge can electrically break down the chemicals comprising the air. Some of the chemical species can react with the PDMS surface to

oxidize it and make it less hydrophobic. After the surface treatment, both surfaces are brought into conformal contact and put aside for one day. An irreversible bonding is formed. Additionally, the plasma treatment facilitates the flow of fluid inside the PDMS channel because its walls are no longer the hydrophobic but are changed to be hydrophilic. Some silanol groups are also generated on the sidewall surfaces of the PDMS channel under the exposure of O_2 plasma, which provides an electroosmotic effect on the sidewalls of the microchannel. The fabricated PDMS device bonded to a cover slip is shown in Fig. 3.14.

3.3 Experimental results and discussions

Figure 3.12: Schematic of the microfluidic chip for droplet generation and 1-D droplet grating formation.

The design of the tunable grating using multiphase droplets in a microfluidic chip is shown in Fig. 3.12. Two immiscible liquids flow into the microchannel from two inlets and form an interface at the T-junction. Here, the immersion oil is used as the carrier liquid and the CaCl_2 solution as the dispersed liquid for droplets. The dynamics of break-up arises from the pressure drop across the emerging droplet. The formed droplets flow along the microchannel and form a 1-dimensional droplet array. When placed light source above the chip, this droplet array will effectively work as a phase grating consisting of alternated regions of immersion oil and CaCl_2 droplets, each with different refractive indices n_{oil} and $n_{droplet}$, respectively.

Figure 3.13: Schematic of the experimental setup.

The measurement system, shown in Fig. 3.13, consists of a halogen light source (He-Ne laser with wavelength 632.8 nm, or HL-2000-FHSA, Ocean Optic), a collimating lens, the optofluidic chip, a collecting lens and a charge coupled device camera (DP70, Olympus). Two immiscible liquids are injected by syringe pumps (NE-1000, New Era) into the microfluidic chip. An optical single-mode fiber through the collimating lens is used to couple the incident light beam onto the droplet grating region. When light propagates through the grating region, the diffraction pattern of the output light is captured by the CCD camera.

3.3.1 Multiphase microdroplet generation

Figure 3.14: Micrographs of the generation of droplets and the formation of droplet array.

Figure 3.14 shows the generation process of droplets. At the T-junction, the injection of CaCl_2 solution causes the interface between the two immiscible flows to penetrate into the main channel. As a result, the CaCl_2 solution enters the main channel and gradually occupies the whole cross-section of the main channel. The immersion oil encounters higher resistance and builds up the pressure until it breaks the neck of the elongated plug. The plug moves downstream freely while the tip of the CaCl_2 solution retracts, waiting for the generation of next plug. When the generated plug flows down to a sudden expansion of the microfluidic channel, it is transformed into a circular droplet.

By coordinating the flow rate of two immiscible liquids, the droplets have diameter ranging from 20 to 50 μm . The period can simultaneously range from 10 to 50

μm . The structure of the droplet grating can be dynamically and reversibly tuned with switching

Figure 3.15 (a) shows the single droplet diameter changes almost linearly with the times of less than two seconds by changing the rates of flow applied to the device. Variances of the continuous oil phase, the grating pitch changes according to the droplet diameter simultaneously.

Furthermore, the stability and the monodispersity of the droplets are investigated. The standard deviations of the droplet size and grating period under certain flow rate ratio are measured and calculated as lower than 5% which means highly uniform and monodisperse. This means that it is easy to form an array of droplet with constant size and period in the microchannel. The whole liquid grating system exhibits high stability over hours when properly designed and operated, as shown in Fig. 3.15 (b).

(a)

(b)

(c)

(d)

(e)

Figure 3.15: The tuning of droplet size and grating period by microfluidic control: (a) Relationship between the size of single droplet and flow rate of the continuous oil phase and (b)-(e) Different droplet sizes and grating pitches achieved by tuning the flow rate.

3.3.2 Diffraction pattern

Figure 3.16 shows the diffraction patterns generated by the droplet grating when using a 632.8 nm He-Ne laser as monochromatic incident source. The upper panels show the simulation results and the lower panels show the experimental results. They show the irradiance distribution for a droplet array grating diffraction pattern of different droplet diameters and grating periods. The period of the circular pattern becomes smaller when the single droplet diameter is larger. The period of the vertical pattern becomes larger and larger when the grating pitch is smaller. When the size of the droplet is close to the incident light wavelength, the diffraction pattern can be clearly seen.

Figures 3.17 and 3.18 show the relationship between the diffraction pattern periods and the droplet grating characteristics. The circular first order period of the circular pattern ranges between 5 and 20 when the droplet diameter value ranges between 10 μm to 20 μm . The vertical pattern period varies between 1.5 to 4.5 when The grating period value ranges between 20 μm and 50 μm . The experimental results agree well with the theoretical calculation curves.

Figure 3.16: Optical micrographs of the diffraction pattern of one-dimension droplet array with different grating period using monochromatic incident light (a-c) Simulation results and (d-e) Experimental results.

Figure 3.17: Relationship between the single droplet diameter and circular pattern period.

Figure 3.18: Relationship between the grating pitch and vertical pattern period.

3.3.3 Droplet optofluidic refractometer

Figure 3.19: Relationship between the refractive index and the modified diffraction efficiency of 0th and 1st order.

Figure 3.19 shows the change of intensities of the Airy disk and the 1st ring at different values of $n_{droplet}$. At $n_{droplet} = 1.340$, the 1st ring reaches its maximum while the 0th order Airy disk has very low intensity. With the increase of $n_{droplet}$ to 1.352 and 1.357, the 1st ring becomes dimmer and the Airy disk grows brighter. At $n_{droplet} = 1.362$, the 1st ring nearly vanishes and the Airy disk is at its maximum. The measured intensities of the 0th order and the 1st order are compared with calculated data. It can be seen that for the 0th

order diffraction, they match well with each other except for those at very low transmission level. This is because the scattering from the other parts (like microchannel edges) introduces a background.

In Fig. 3.19, the transmission efficiency varies almost linearly over the refractive index range $n_{droplet} = 1.344 - 1.352$. Therefore, the whole system can be used as an optofluidic refractometer. Simply using software to analyze the diffraction pattern and the intensity distribution can give more accurate data of the droplet refractive index. The CCD camera used to record the diffraction pattern has a normalized intensity level range from 0 to 255. A total $n_{droplet}$ change of 0.016 refractive index unit (RIU) from 1.341 to 1.357 is measured within this intensity level range. The definition of refractive index unit (RIU) is to distinguish a number as referring to a refractive index. It is useful for expressing quantities vary with refractive index. RIU is used for determine the performance of refractometers.” Therefore, the detection limit (change of RIU per unit change of intensity level) of the system is 6.27×10^{-5} RIU. The channel height and the CCD camera resolution are the main factors that affect the detection limit.

Figure 3.20: Diffraction pattern of 0th and 1st order for different droplet refractive indices.

3.3.4 Droplet grating based transmission color selective system

For white incident light, the color patterns of the 0th diffraction order are shown in Fig. 3.21. The increase of $n_{droplet}$ from 1.439 to 1.447 causes the color to vary from magenta to red, which is a clear manifestation of the color filtering effect. With the change of the refractive index of the CaCl₂ solution, the central wavelength of the filtered color is shifted at a rate of ~ 50 nm/RIU.

The comparison of the measured colors and the predicted values are presented in the CIE 1931 xyY color chart as shown in Fig. 3.22(c). It is noted that the non-uniform spectral power distribution of the light source is already considered in the simulation. In the CIE chart, a rough matching is found between the chromaticity points. Moreover, the obtained colors are scattered around and well separated from the white point D65 (6500K). It is well demonstrated that the color filtering property can be tuned by changing the refractive index of droplets with high sensitivity and stability.

Figure 3.21: Optical micrographs of the diffraction pattern of one-dimension droplet array with different grating period using polychromatic incident light (a) $d = 47.3 \mu\text{m}$ (b) $d = 28.0 \mu\text{m}$ and (c) $d = 23.1 \mu\text{m}$.

(a)

(b)

Figure 3.22: Measured diffractions of white incident light when the droplet grating is used as a 0th order color filter (a) Color patterns (b) SPD of light source and (c) Measured chromaticity values of the subtractive colors as compared to the predicted

3.4 Summary

This chapter focuses on an optofluidic grating that is reconfigurable using multiphase droplets on a microfluidic chip, which has the advantages of high stability and wide tunability in droplet size, grating period, and refractive index, making it promising for biochemical and bio-material applications.

To control the multiphase fluids which are more complicated compared to a single-phase flow, various physical phenomenon in microfluidic devices are analyzed. The formation process of droplets in the microfluidic T-junction is described. At low capillary numbers, the dominant contribution of the dynamics break-up arises from the pressure drop across the emerging droplet. A simple scaling relation predicts the size of droplets produced in T-junctions over a range of rates of flow of the two immiscible phases, the viscosity of the continuous phase, the interfacial tension and the geometrical dimensions of the device.

The microfluidic chip is fabricated in PDMS using soft-lithography. The PDMS chip is made by molding technology, where using SU-8 development to do the master patterning. The patterned PDMS slab is then bonded with a glass cover slip by plasma bonding with a corona treater. The transparency and low absorption of the PDMS allow for the integration of the optical detection system into the chip.

A reconfigurable optical diffraction grating is designed and fabricated using multiphase droplets on a microfluidic chip. The uniform and evenly-spaced circular droplets are generated by continuously dispersing two immiscible liquids into a T-

junction to produce plugs, which are then transformed into a circular shape at a sudden expansion of microchannel. It works with high stability and tunability in grating period and refractive index variation to obtain various diffraction patterns. A phase-transmission-grating-based compact optofluidic refractometer has been realized based on the droplet array grating system; the detection limit for the refractive index change is around 6.27×10^{-5} . The droplet array grating can also work as a zeroth-order transmission color filters, and in our test, four primary colors have been successfully selected from a white incident light source. Such grating has the advantages of high stability and wide tunability in droplet size, grating period and refractive index, making it promising for biochemical and biomaterial applications.

CHAPTER 4

BACTERIOPHAGE DETECTION BY DROPLET-BASED OPTOFLUIDIC SYSTEM

This chapter focuses on the design and fabrication of a droplet-based optofluidic system to detect bacteriophages via measuring the growth of the host cell infected by the bacteriophages in a microdroplet carrier. The growth of the host cell, *Escherichia coli* (*E. coli*), is detected by the scattering pattern of the microdroplet carrier and the pattern is analyzed using the mean power frequency. Based on the relationship between mean power frequency and the concentration of the *E. coli* in a microdroplet carrier, the increment of a single *E. coli* can be detected between 0 to 20 *E. coli* cells in one droplet while the increment of 2 *E. coli* can be detected from 21 to 100 *E. coli* cells. The growth curve of *E. coli* cultured individually or co-cultured with λ bacteriophages or M13 bacteriophages is further portrayed using the detection method. The water sample containing λ bacteriophages is measured using the droplet-based optofluidic system. The water sample with the bacteriophage concentration of 10^3 pfu/ml and 10^4 pfu/ml is detected and the result is in accordance with the calculated expectant. This breakthrough optofluidic technology creates a new real-time, label-free and high sensitivity method for bacteriophages detection.

4.1 Droplet-based microfluidic system for bacteriophage detection

4.1.1 Principle of droplet-based microfluidic detection

The principle of the droplet-based optofluidic system for bacteriophage detection via the growth of host cell is shown in Fig. 4.1. The traditional method of plaque assay for bacteriophage detection is a widely used approach in determining the quantity of virus [173]. The water sample is diluted to a proper bacteriophage concentration and the sample is inoculated onto the host cell grown agar plate. After an incubation period, the bacteriophage infects the host cell and releases their progenies. The spread of the new bacteriophages is restricted to neighboring cells by the agar gel, which will form a circular zone of infected cells called a plaque. This method requires a long period of processing time as well as an accurate pre-estimation of the sample concentration. Each agar plate can detect only 1 ml of water sample, which is not practical for large volume detection. For the droplet-based optofluidic system for bacteriophage detection, the water sample is first mixed with *E. coli* culture medium and then divided into a large number of droplets by using microfluidic droplet generation, which allows high generation rates of monodisperse droplets. As the microdroplet flows into the microchannel, the content in the microdroplet is mixed rapidly and facilitates the infection of the host cell with the bacteriophage.

The cell in a microdroplet containing λ bacteriophages will most probably undergo a lysis cycle. The phages identify the host cell *E. coli* by binding on the surface. Then the phage deoxyribonucleic acid (DNA) is ejected from the phage into the cell and duplicated inside the *E. coli*. After an incubation period, the λ bacteriophage DNA is

replicating repeatedly and a multiple of new bacteriophages are assembled inside the host cell. Finally, the host cell is lysed, releasing the assembled λ bacteriophages and other cell contents into the extracellular environment [174]. As a result, the host cell growth rate in the bacteriophage containing microdroplet will be significantly reduced compared with the one with only a normal (non-infected) host cell. The final concentration of host cells will then affect the scattering pattern of the microdroplet carrier, which is used to detect the existence of the bacteriophage. Due to the better mixing condition and the higher contact ratio between bacteriophages and host cells, the infection is more effective and efficient than the conventional plaque assay. In addition, no pre-treatment is needed, making the optofluidic detection much more convenient and improving the detection accuracy.

Figure 4.1: Schematic illustration of optofluidic chip for bacteriophage detection that consists of: (a) Water sample containing bacteriophage and *E. coli* in culture medium are split into microdroplets by microfluidic structure, (b) Two microdroplets merge together and water sample is mixed with host cell *E. coli*, (c) Host cell growth condition in the microdroplet is affected by the presence of the bacteriophage and (d) Host cell growth condition is detected by using optical detection system.

4.1.2 Optofluidic system design and fabrication

Figure 4.2: Schematic of the optofluidic chip.

The structure of the optofluidic chip is shown in Fig. 4.2. The sample containing bacteriophages is mixed with another liquid with a high concentration (5×10^8 cell/ml) of host cells in the microfluidic channel. Then the mixed solution is divided into microdroplets using the mineral oil (Sigma, USA) as continuous flow. As the droplet flows into the microchannel, the content in the droplet is mixed rapidly facilitating the infection of the host cells by the presence of bacteriophages. The microdroplets containing both the bacteriophages and the host cells are incubated in a microchamber with mineral oil extraction outlet, which helps to store a large number of droplets. After the incubation period, the valve towards downstream optical detection area is opened and the incubated droplets go through the light scattering area successively. The microfluidic chip is fabricated in polydimethylsiloxane (PDMS) material using standard soft lithography processes. The triple-layered PDMS slab is then bonded to a coverslip by exposing to air plasma for 15 s using a handheld corona treater (BD-25, Electro-Technic Products). The coverslip is

Figure 4.3: Optical setup of the optofluidic detection system.

further bonded to a glass slide with a thin layer PDMS coating for better robustness in operation. The optical setup of the optofluidic system is shown in Fig. 4.3. The light source is He-Ne laser ($\lambda = 632.8$ nm, 800 mW, Thor Lab, USA). The beam is reflected by a beam splitter and focused on the micordroplets at the detection area in the microfluidic chip as shown in Fig. 4.3. The light scattered from the droplets is collected at a distance of 80 mm with a CCD line-scan camera (Nikon digital sight DS F-11) and pass to the signal processor for the further analysis.

4.1.3 Relationship between bacteriophage amplification and host cell growth

Figure 4.4: The transmitted optical density of *E. coli* cultured individually or co-cultured with λ phages and M13 phage.

The *E. coli* (*E. coli* K12, Biolabs, USA) is cultured as host cell. A small portion of the *E. coli* powder is transferred to a sterile pipette tip with liquid Luria Broth medium (Sigma, USA) and incubated in a water bath shaker with 200 rpm at 37°C for 8 hours. A drop of liquid culture is spread on a selective agar plate and propagated overnight at 37 °C. After the formation of the *E. coli* colonies, a single colony of *E. coli* is selected and transferred to liquid Luria Broth medium to grow overnight. The final cultured *E. coli* concentration is measured using OD₆₀₀ with spectrometer (PG Instruments T60 visible spectrophotometer). The bacteriophages we used are λ phage (Biolabs, USA) and M13 phage (Biolabs, USA). The bacteriophages are diluted with Tris buffered saline (Sigma,

USA) and amplified using *E.coli* as the host cell for preparation. The bacteriophage sample concentration is measured by plaque assay method [175]. The λ bacteriophage most time goes through the lytic cycle most of the time. When the λ bacteriophage recognizes the host cell, the phage binds to its host cell and Deoxyribonucleic acid (DNA) containing in the head of the phage is ejected through the tail into the cytoplasm of the host bacteria cell. The λ bacteriophage DNA is replicated many times and a multiple of new bacteriophages are assembled inside the host cell. Finally, the host cells lysis, releasing the assembled λ bacteriophage and other cell contents into the environment [116]. The M13 bacteriophage is a kind of non-lytic virus. Infection with M13 bacteriophage is not lethal to the host cell. However, the infection causes turbid plaques in *E. coli* due to the transfer and combination of phage to host cell DNA, which will induce a decrement in the rate of host cell growth [176].

The optical density at the wavelength of 600 nm (OD600) of *E. coli* cultured individually or co-cultured with λ phage and M13 phage in culture flask, which is the general measurement method of cultured bacteria concentration, and the results are shown in Fig. 4.4. The optical density is the logarithmic ratio of the incident light intensity falling upon the *E. coli* culture medium to the transmitted through the medium and it is correlated with the *E. coli* concentration. The *E. coli* growth is expressed as the change in the number of viable cells with time lapse. The *E. coli* cultured individually exhibits as a typical bacterial growth curve [177]. Initially, during the lag phase the bacteria adapt themselves to the new environment during the first hour. During this period, the individual bacteria are maturing but not yet able to divide. After the first hour is the exponential phase, the number of the bacteria doubles per unit time due to cell division. The third period is the stationary phase after 6 hours due to the growth limiting

factors, the most important living the depletion of nutrient. When co-cultured with λ bacteriophages, the infection process of the *E. coli* with λ bacteriophages lasts for approximately 2 hours. After the second hour, the population of the *E. coli* cells decreases obviously since most of the bacteria are infected by the phage after several lysis cycles and finally break up during the phage release period. When co-cultured with M13 bacteriophages, after infection, the bacteriophages go through the lysogenic cycle. There is not significant reduction in the population of the host cell. However, due to the influence of the existence of the bacteriophage, the speed of growth of the *E. coli* host cell is slowed down. Compared to the normal individual culture, the final concentration of the M13 bacteriophage infected host cell after 7 hours co-culture is merely half of the normal value for non-infected cells.

4.2 Droplet scattering pattern

4.2.1 Scattering pattern

Figure 4.5: Schematic illustration of the optofluidic imaging of droplets encapsulated with co-cultured bacteriophage and host cells, *E. coli*.

The scattering patterns of microdroplets with the same diameter $D = 60 \text{ }\mu\text{m}$ but different concentrations of inclusion are recorded and collected as shown in Fig. 4.5. The average size of the inclusion *E. coli* is a rod with $d = 0.5 \text{ }\mu\text{m}$ and $l = 2 \text{ }\mu\text{m}$. For the microdroplet without inclusion, the scattered light shows pure Mie fringes. For the case of a microdroplet with inclusions, the scattering light from droplet would be reflected and refracted by the inclusions and the resulting scattering pattern is a coherent superposition of scattering light from the microdroplet and those from the inclusions, which results in regular Mie fringes decorated by random speckles. As the inclusion concentration increases, the scattering pattern deviates much significantly from clear Mie fringes, and there are more contributions from the high frequency speckle signal [178].

Fig. 4.6 shows the scattering patterns of microdroplets containing different concentration of *E. coli*. In this experiment, the diameter of the microdroplets is controlled and fixed at $d = 60 \text{ }\mu\text{m}$. The diffraction pattern of the microdroplet containing *E. coli* is a combination of single circular aperture Fraunhofer diffraction by the shape of the droplet and the scattering signal of the content inside the droplet. The circular diffraction pattern appearance is determined by the diameter of the single droplet. The pattern intensity distribution is determined by the refractive indices of the two immiscible liquids: the immersion oil with the refractive index of $n_{oil} = 1.467$, and the mixture of culture medium including *E. coli* and bacteriophage, which is defined as effective refractive index $n_{droplet}$. When $n_{droplet}$ of the microdroplet containing pure culture medium without any *E. coli* is 1.3331, the $n_{droplet}$ will increase as the concentration of *E. coli* increases. The diameter of the microdroplets can be controlled by regulating of the flow rate of the two immiscible liquid. Using the same microfluidic device with the known structure geometry, the diameter of the microdroplet is constant when the flow rate is unchanged, which is independent of the viscosities and the interfacial tensions of the two immiscible liquids.

The stability and the monodispersity of the microdroplets are also investigated. The diameter of 1000 microdroplets forming under a certain ratio of the 2 liquids flow rates is measured and the standard deviation of the droplet diameter is calculated, and found to be lower than 3%. This means that our system can reliably provide highly monodisperse and very stable microdroplet formation in the microfluidic chip.

When the diameter of the droplet is constant, the pattern distribution is mainly influenced by the content of the droplet. The volume ratio of a single *E.coli* to the single microdroplet carrier is lower than 0.01%. Therefore, the bacteriophage volume can be

Figure 4.6: The scattering pattern of microdroplets containing different concentration of *E.coli* (a) 10^9 cell/ml (b) 5×10^8 cell/ml (c) 2×10^8 cell/ml (d) 10^8 cell/ml (e) 5×10^7 cell/ml (f) 2×10^7 cell/ml (g) 10^7 cell/ml and (h) Control group.

nearly neglected due to very small picoliter size [3]. The effective refractive index of the mixture changes only very slightly for droplets carrying a very high concentration of bacteria (10^9 cell/ml, approximate 100 cell/droplet) as compared to that droplet with very low concentration (10^7 cell/ml, approximate 0-1 cell/droplet).

However, the scattering pattern is significantly influenced by the concentration of the cells due to multiple reflections and refractions of the light incident onto the droplet. In this experiment, the incident light wavelength is constant ($\lambda = 632.8$ nm), and the inclusion particles inside the microdroplet carrier (the *E. coli* cells) have a monodisperse size which is comparable to the incident wavelength. The Mie scattering intensity is

mainly determined by the concentration of the *E. coli* cells. As the concentration of inclusion increases from low value concentration (10^7 cell/ml, approximate 0-1 cell/droplet) to very high value (10^9 cell/ml, approximate 100 cell/droplet), the resultant scattering pattern evolves gradually into a disordered one. The clear fringe signal is nearly covered by the scattering signal at a high concentration of bacteria (10^9 cell/ml, approximately 100 cell/droplet) as shown in Fig. 4.7(a), which is because the high frequency speckle signal, predominantly from inclusions, would blur the clear fringe pattern.

4.2.2 Mean power frequency analysis

Figure 4.7: Scattering pattern analysis with mean power frequency of microdroplets containing different concentration of *E. coli* (a) The scattering pattern and intensity distribution of *E. coli* with concentration of 10^9 cell/ml (100 cell/droplet) and (b) The scattering pattern and intensity distribution of *E. coli* with concentration of 10^7 cell/ml (0-1 cell/droplet).

Consider the Fast Fourier Transform (FFT) for the frequency spectrum of the scattering patterns, which is expressed as

$$IF = \sum_{u=1}^M \sum_{v=1}^M Is(x, y) \exp\left(-i \frac{2\pi ux}{M}\right) \exp\left(-j \frac{2\pi vy}{M}\right) \quad (4.1)$$

where I_s is the two-dimensional distribution of the scattering light. The power spectrum is

$$\phi(f) = IF * IF \quad (4.2)$$

where f is the frequency of intensity variation. The mean power frequency is the normalized first order power spectral moment. It can be expressed as

$$MPF = \frac{\int_0^\infty f\phi(f)df}{\int_0^\infty \phi(f)df} \quad (4.3)$$

where f is the frequency of intensity variation. This mean power frequency is used to analyze the variation of frequency of the scattering pattern.

Scattering pattern analysis with mean power frequency of microdroplets containing different concentration of *E. coli* is shown in Fig. 4.7. Comparing the two groups of graphs from high to low *E. coli* concentrations, it can be seen that at the higher concentration, the scattering pattern of the whole microdroplet has higher level of disorder. The intensity distribution of the same order of the droplet diffraction pattern splits into more individual peaks. The FFT pattern shows higher non-uniform frequency distribution. The mean power frequency value of the single microdroplet scattering pattern increases with the incensement of the concentration of *E. coli* in the microdroplet as shown in Fig.4.8. From 0 to 100 cells per each microdroplet, the mean power frequency value increase nearly two-fold. Based on this curve, the number of *E. coli* can be calculated from the scattering pattern of a microdroplet containing unknown concentration of host cells. The rate of increase is quite high and near linear growth from 20 to 100 cells per droplet which can guarantee high resolution of the measurement method. For the nonlinear region from 0 to 20 cells per droplet, more data is measuring to

Figure 4.8: Mean power frequency changed by the *E. coli* concentration in the microdroplet.

ensure for an accurate estimation. The unknown concentration of host cells is estimated based on the curve and Equation 4.3 on page 101. The detection resolution is mainly limited by the quality of the scattering pattern captured. With the present system the increment of a single cell can be detected between 0 to 20 cells and the increment of 2 cells can be detected from 21 to 100 cells as the detection of 5% cell population change. The detection performance can be further improved by using a CCD camera with higher speed and sensitivity.

4.2.3 Principal component analysis

Principal component analysis (PCA) is a way of identifying patterns in data, and expressing the data in such a way as to highlight their similarities and differences. It is a useful statistical technique that has found applications in fields such as face recognition and image compression, and is a commonly used technique for finding patterns in data of high dimension. The central idea of PCA is to reduce the dimensionality of a dataset consisting of large number of interrelated variables, while retaining as much as possible of the variation present in the dataset. This is achieved by transforming to a new set of variables, the principle components (PCs), which are uncorrelated, and are ordered so that the first few retain most of the variation present in all of original variables [179-181].

Many of the mathematical and statistical properties of PCA have been discussed based on a known population covariance (or correlation) matrix. As well as being driven from a statistical viewpoint, PCA can be found using purely mathematical arguments. They are given by an orthogonal linear transformation of a set of variables optimizing certain algebraic criterion. The process of PCA analysis for the photographic data collected from a batch of experiments is as followed. The first step needed to perform a principal component analysis is performed on a set of data to collect and choose a batch of data. Then, the mean from each of the data dimensions are subtracted. The covariance matrix and the eigenvectors and eigenvalues of the covariance matrix are calculated. Finally the components are chosen to form a feature vector, which can be used to divine a new set of data/

Figure 4.9 Scattering pattern analysis with principal component analysis (PCA).

Figure 4.9 shows the principal component analysis results of the scattering patterns of single microdroplets containing different concentration of *E. coli*. The pattern is analysed using Matlab to collect and calculate the data. Results show that the scattering patterns, in general, can be separated by choosing suitable parameters for PCA analysis.

4.3 Bacteriophage detection via host cell growth

4.3.1 Co-culture of *E.coli* with λ bacteriophage

Figure 4.10 The transmitted optical density of *E. coli* culture.

The concentration varies of *E. coli* cultured individually or co-cultured with λ phages or M13 phages in the droplet which is measured based on the relationship between *E. coli* cell number in the single microdroplet and the mean power frequency of the scattering pattern of the microdroplet. For comparison convenience, the number of *E. coli* is also expressed by the OD600 value, which is calculated using the known microdroplet diameter and measured dependence between *E. coli* concentration and OD600. Fig. 4.10 shows the comparison between the *E. coli* cultured individually in the microdroplet and the *E. coli* culture using flask. The red line shows the condition of culture using flask as the traditional method while the black line shows the condition of

Figure 4.11 The transmitted optical density of *E. coli* co-culture with λ phage.

culture in the microdroplet. The growth of *E. coli* cultured individually in the microdroplet still follows the typical bacterial growth curve. However, under a confined microenvironment in the closed droplet, the metabolic waste of the *E. coli* cannot be removed and the nutrition is limited without refreshment. The speed of growth of the *E. coli* is obviously slower than in the traditional flask culture. The gap of the growth speed grows larger at higher concentrations as the nutrition falls more drastically when the number of *E. coli* cells is large.

When the *E. coli* is co-cultured with λ bacteriophages in the microdroplet, the initial infection period is shorter than in the traditional method in flask, as shown in Fig. 4.11. The start time point of the obvious decrement of the population of *E. coli* occurs earlier. It is mainly because at the beginning period of first to second hour, the bacteriophage number is quite low. In the droplet picoliter microenvironment, the contact

probability between λ bacteriophage and its host cell of *E. coli* is which higher than in a large open environment. During the travelling of the microdroplet in the microchannel, the well mixing of the content further promote the infection speed.

4.3.2 Co-culture of *E. coli* with M13 bacteriophage

Figure 4.12: The transmitted optical density of *E. coli* co-culture with M13 phage.

As shown in Fig. 4.12, when the *E. coli* is co-cultured with M13 bacteriophage, the growth speed of the M13 bacteriophage infected *E. coli* is slowed down further due to the same reason as demonstrated earlier. Using the optofluidic detection system, a real-time and label-free measurement of the concentration of the *E. coli* inside the microdroplet is achieved. The three conditions in the flask are also met in the microdroplet.

4.3.3 λ bacteriophage detection

To verify the detection ability of the optofluidic system, water samples containing different concentrations of bacteriophage are measured and the results are shown in Fig. 4.13. For the microdroplet with fixed diameter of $d = 60 \text{ }\mu\text{m}$, the volume is approximately 10^{-7} ml . The water sample with known bacteriophage concentration is injected into the optofluidic system. The water sample containing bacteriophage is mixed with *E. coli* culture medium and divided into a large number of microdroplets. The initial concentration of the cultured *E. coli* is $5 \times 10^8 \text{ cell/ml}$ (50 cell/droplet), which is a suitable environment for the λ bacteriophage infection. After 4 hours of storage and incubation in the microculture chamber, the microdroplets are released and passed through the optical detection area. The scattering pattern of each droplet is recorded and analysed. The host cell *E. coli* will be infected in the microdroplets containing λ bacteriophage and the scattering pattern of these microdroplets will have lower mean power frequency value. When this type of scattering pattern is detected, a positive signal is counted. The microdroplets can be divided into a series of testing groups, with each group size of N microdroplets. The positive signal number of each testing group f can be expressed as [182]

$$f = C_{bacteriophage} \times V \times N \quad (4.4)$$

where $C_{bacteriophage}$ is the λ bacteriophage concentration of the water sample and V is the volume of a single microdroplet. Fig. 4.13 shows the signal recording of 10 groups of microdroplets with a group size of 1×10^5 droplets. The water samples have known bacteriophage concentration of $C_{bacteriophage} = 10^3 \text{ pfu/ml}$ and $C_{bacteriophage} = 10^4 \text{ pfu/ml}$, respectively. The average positive signal number of $C_{bacteriophage} = 10^3 \text{ pfu/ml}$ and

Figure 4.13: Signal recording of λ phage containing sample with the concentration of 10^3 pfu/ml and 10^4 pfu/ml.

$C_{bacteriophage} = 10^4$ pfu/ml is 1 and 10, respectively, which is corresponding to the theoretical calculation. As a result, the optofluidic system has realized a fast and reliable detection of the bacteriophage. The optofluidic system can detect a lower concentration of bacteriophage by increasing the number of microdroplet in each group. The dominant limitation of the resolution is the stability of the signal processing system.

4.4 Summary

This chapter focuses on the design and fabrication of a droplet-based optofluidic system to detect bacteriophages, which is co-cultured with the host cell *Escherichia coli* (*E. coli*) growth in a microdroplet carrier. The growth of the host cell is detected by the scattering pattern of the microdroplet carrier and the pattern is analyzed by using the mean power frequency. The growth curve of *E. coli* co-cultured with λ bacteriophage is portrayed by using this detection method.

The bacteriophages are first mixed with the host cell *E. coli* culture medium and the mixture is then divided into a large number of microdroplets. After an incubation period, the host cell growth in the bacteriophage-contained microdroplets should be obviously different from the microdroplets containing only the host cells. The final concentration of the host cell affects the scattering pattern of the microdroplet, which can be used to detect the existence of the bacteriophage.

The mean power frequency value of the single microdroplet scattering pattern is calculated. It increases with the increment of the concentration of *E. coli* in the microdroplet. Based on this curve, the number of *E. coli* can be deduced from a measured scattering pattern of a microdroplet containing an unknown concentration of host cells.

The concentration varies of *E. coli* can vary, depending on whether it is cultured individually or co-cultured with λ phage in the droplet. The number of the latter is computed based on the relationship between the number of *E. coli* cells in the single microdroplet and the mean power frequency of the measured scattering pattern of the microdroplet. The results show that the growth speed of the *E. coli* is slower than that for the traditional flask culture. The start point of the decrement of *E. coli* population occurs

earlier than in cultured of uninfected cells. The water sample containing different concentration of λ bacteriophages is measured by the optofluidic system, and the measured results agree with the analysis results provided by the theoretical calculation.

In this chapter, the λ bacteriophage co-cultured with the host cell *E. coli* is detected by using a droplet-based optofluidic system. The system provides a means of a real-time, label-free and high sensitivity quantification of bacteriophage through the measurement of host cell growth with high accuracy and stability.

CHAPTER 5

STUDY OF ENDOTHELIAL CELL DYSFUNCTION USING MICROFLUIDIC CHIP

This chapter presents how a hemodynamic microfluidic chip system is developed and used to better understand how hyperglycemia induces endothelial cell dysfunction under diabetic conditions. The microfluidic system is used to study the production of reactive oxygen species (ROS) and apoptosis of endothelial cells grown in the microchannel under different physical (shear stress) and chemical (glucose concentration) condition. The elevated ROS level in the endothelial cells is measured and the morphological change of mitochondria from filamentous reticular networks to diffused and short fragments is observed. The system also combines a caspase-3 based fluorescence resonance energy transfer (FRET) biosensor cell line which detects endothelial cell apoptosis in real-time, in a post-treatment effect and limited cell sample. As a result, it can be effectively be used provide new insight on how glucose causes endothelial cell dysfunction, which is the major cause of diabetes-derived complications.

5.1 Hemodynamic Lab-on-chip system for Endothelial cell study

5.1.1 Endothelial cell response to physical and glucose stimulations

Figure 5.1: Blood vessel structure and function of endothelial cells.

Blood vessels are the part of the circulatory system that transports blood throughout the body. There are three major types of blood vessels: arteries, capillaries and veins. The arteries carry blood away from the heart. The capillaries enable the actual exchange of water and chemicals between the blood and the tissues and the veins carry blood from the capillaries back toward the heart. The arteries and veins have three layers: the tunica intima, the tunica media and the tunica adventitia as shown in Fig. 5.1. The tunica intima is a single layer of endothelial cells glued by a polysaccharide intercellular matrix, surrounded by a thin layer of sub-endothelial connective tissue. The tunica media is the circularly arranged elastic fiber, connective tissue, polysaccharide substances, the

second and third layer are separated by another thick elastic band called external elastic lamina. The tunica media is rich in vascular smooth muscle, which controls the caliber of the vessel. The tunica adventitia is entirely made of connective tissue. It also contains nerves that supply the vessel as well as nutrient capillaries in the larger blood vessels. The capillaries consist of little more than a layer of endothelium and occasional connective tissue. For all three types of blood vessels, the endothelium belong to the tunica intima is the thin layer of cells that lines the interior surface of blood vessels and lymphatic vessels, forming an interface between circulating blood or lymph in the lumen and the rest of the vessel wall. The cells that form the endothelium are called endothelial cells. Endothelial cells in direct contact with blood are called vascular endothelial cells, whereas those in direct contact with lymph are known as lymphatic endothelial cells.

Figure 5.2: Schematic illustration of a bio-model showing how endothelial cells interact with their physical and biochemical microenvironments.

Blood vessels play a huge role in virtually every medical condition. Cancer, for example, cannot progress unless the tumor causes angiogenesis to supply the malignant cells' metabolic demand. Atherosclerosis, the formation of lipid lumps in the blood vessel wall, is the most common cardiovascular disease, the main cause of death in the Western world. Blood vessel permeability is increased in inflammation. Damage, due to trauma or spontaneously, may lead to hemorrhage due to mechanical damage to the vessel endothelium. In contrast, occlusion of the blood vessel by atherosclerotic plaque, by a blood clot or a foreign body leads to downstream ischemia and possibly necrosis. Vessel occlusion tends to be a positive feedback system; an occluded vessel creates eddies in the normally laminar flow or plug flow blood currents. These eddies create abnormal fluid velocity gradients which push blood elements such as cholesterol or chylomicron bodies to the endothelium. These deposit onto the arterial walls which are already partially occluded and build upon the blockage.

Diabetes mellitus is a disease with worldwide high morbidity and mortality, which is a serious threat to human health. A wide variety of studies suggest that hyperglycemia is the primary cause of the development and progression of abnormal vascular growth and the complications of diabetes, whose major risk factor is the endothelial cells dysfunction under high glucose concentration. Another important factor, shear stress, which is imposed tangentially to the cell surface caused by the pulsatile hemodynamic forces in the blood vessels, also has complex influence to endothelial growth. In the designed Lab-on-a-chip system, shear stress generated by the laminar flow is directly imposed onto the endothelial cells' surface and high concentrations of glucose are added into culture medium to mimic the physical and chemical stresses applied to the cells to mimic hyperglycemic conditions in diabetes patients as shown in Fig. 5.2. The combination of these abnormal microenvironments may promote intracellular ROS generation, which can in turn overload the antioxidant defense system of the cells, leading to the endothelial cell dysfunction through the caspase-3 activation pathway [183]. A novel bio-sensing assay based on caspase-3 activity, which can detect apoptosis-activating compounds in a high throughput manner with a genetically engineered cell line, is integrated into the platform. The platform can achieve a real-time in vitro and convenient way for cell dysfunction detection.

5.1.2 Design of the microfluidic system

(a)

(b)

Figure 5.3: Schematic illustrations of: (a) The design of the microfluidic chip and (b) The hemodynamic Lab-on-a-chip system with configurable flow profile to mimic the blood flow in blood vessel.

The design of the hemodynamic Lab-on-a-chip system is shown in Fig. 5.3. The chip structure consists of three layers: (1) a bottom layer with a microfluidic

network for cell loading and liquid injection, (2) a middle layer with pneumatic valves and (3) a top layer with pneumatic connecting channels to control a series of pneumatic valves simultaneously. The microfluidic chip is fabricated in polydimethylsiloxane (PDMS) using a standard soft lithography process [184]. The triple-layer PDMS slab is then bonded to a cover slip using plasma bonding.

The microfluidic network consists of two inlets connected to a concentration gradient network with three microchannels branches. Each branch of microchannel has 4 cell culture sites to perform a series of time course experiments. Each cell culture site has a width of 600 μm and a height of 150 μm . The downstream of each cell culture site is connected to a common outlet and has a pneumatic valve for flow switching.

Initially, the first three rows of valves are closed and the last row of valves is opened. Therefore, the liquid injected into the inlet can flow through the 4 culture sites at each microchannel branch. In this stage, endothelial cells are loaded, cultured and stained prior to the application of shear stress and chemical treatments. When the cells are ready for the shear stress and chemical treatments, the two inlets are injected with the normal culture medium, the culture medium with 20 mM glucose solution, respectively. With the concentration gradient network, three chemical conditions can be achieved, i.e. normal culture medium, culture medium with 10 mM glucose and culture medium with 20 mM glucose. The first data point is measured at 15 min after the shear stress treatment. At this stage, the valves in the last row are closed and the valves in the other three rows are kept open, as shown in Fig. 5.3(a). Subsequently, the cell culture sites in the last row are disconnected from the microfluidic flow in

which the medium is flowing into the side branch at the downstream of the third row of culture sites to the outlets. Similarly, each row of cell culture sites could be disconnected from the main microfluidic flow at each time frame by controlling the pneumatic valves. With this densely designed microfluidic network, one single chip can perform a series of experiments with different chemical treatments and time course.

A pulsation free precision pump (Nemesys, Cetoni) is used for cell loading and media injection as shown in Fig. 5.3(b). The flow profile of the pump is configured to mimic the blood flow in a blood vessel. A bubble trapper is connected between the precision pump and the microfluidic chip. The bubble trapper could significantly prevent bubbles from flowing into the chip since they can cause the endothelial cells in the microchannel to detach from the cover slip and flow out of the chip. A heating plate is used to maintain the temperature of the microfluidic chip at 37°C for long-term observation under the microscope.

The hemodynamic microfluidic chip system can be further developed as shown in Fig. 5.4(a). The microfluidic chip system consisted of four rows of microchannels; and each microchannel has three microchambers, which serve as the cell culture sites. Each microchamber has a width of 600 μm , a length of 3 cm and a height of 150 μm . The separated microchannels are connected by flexible connectors. The connection condition and sequence of the channels can be easily adjusted, which facilitates conducting both successive operations of fluorescent dye staining and washing and performing parallel experiments under different flow conditions.

Figure 5.4: Design of the microfluidic chip. Schematic illustrations of: (a) Hemodynamic microfluidic chip system and (b) Experimental process to study endothelial cell death under different pulsatile shear stress conditions.

A simple illustration of the experimental process by using a three-row microfluidic chip is shown in Fig. 5.4(b). First, endothelial cells are loaded into the microchip with all the three microchannels being connected in series. After the cells

reached confluence, the culture rows are placed under different pulsatile flow conditions and the flow conditions of each row can be changed without affecting other rows. The flow condition is controlled by a pulsation free precision pump (Nemesys, Cetoni), with a flow profile mimicking the blood flow in the blood vessel. The pulsatile flow profiles have been characterized and verified by tracking the displacement of suspended 5- μm microbeads in the culture medium with a high-speed camera (Photron, FASTCAM SA3). Three flow profiles can be experimented on the endothelial cells simultaneously with three rows of microchannels: (1) a 12-hour pulsatile shear stress condition of 15 dyne cm^{-2} (SS15) with a normal heart rate of 70 beat per minute, which represents a human in resting condition; (2) an 1-hour pulsatile shear stress condition of 30 dyne cm^{-2} (SS30) with a heart rate of 140 b.p.m., which represents a human in exhaustive exercise condition, and subsequently 11 hours SS15; and (3) a 2 hours of SS30 and subsequently 10 hours SS15. Initially, row 1 is connected to the syringe, which exerted a shear stress profile of SS15. Row 2 and 3 are interconnected to the syringe, which exerted a shear stress profile of SS30. After 1 hour, row 2 is disconnected from row 3 and connected to row 1 for SS15. Similarly, after 2 hours, row 3 is connected together with row 1 and 2 for SS15. As a result, all three flow profiles can be easily realized in a single microfluidic chip system.

The number of microchannel rows can be increased when more complex and varying flow condition processes are needed. By using this microfluidic system, a single microfluidic chip can not only perform a series of experiments with different pulsatile flow conditions simultaneously, but also realize the parallel comparison among several cell culture chambers within one experimental condition.

5.1.3 Control on the pulsatile flow condition

Figure 5.5: The volume flow rate profile of plasma in a typical artery with endothelial cells under a shear stress of 15 dyne cm^{-2} .

In this experiment, a typical velocity profile of the artery is chosen to induce the shear stress on the cultured endothelial cells in the chip as shown in Fig. 5.5 [185, 186]. Under normal condition, the typical shear stress in the artery is 15 dyne cm^{-2} and the heart rate is 70 beats per minute (bpm). During exhaustive exercise, the shear stress and the heart rate may be increased to 30 dyne cm^{-2} and 140 bpm, respectively [187]. As the microchannel is fabricated into a rectangular shape, the shear stress in the microchannel is determined as [188]

$$\tau = 12 \frac{Q\mu}{h^2 w} \quad (5.1)$$

where Q is the volume flow rate, μ is the liquid's viscosity, h and w are the height and

width of the microchannel, respectively. As the microchannel has a height of 150 μm and a width of 600 μm , the plasma with viscosity μ of 1.2 mPa requires a volume flow rate of 4.23 $\mu\text{L s}^{-1}$ to achieve a shear stress of 1.5 Pa. Based on Eq. 5.1, the volume flow rate can be increased to achieve higher shear stress. In the experiment, the shear stress is adjusted from 15 to 30 dyne cm^{-2} and the selected heart rates are 70 bpm under normal condition and 140 bpm under exhaustive exercise.

5.1.4 Endothelial cell culture

(a)

(b)

Figure 5.6: Phase images of endothelial cells: (a) seeded in the microchannel and (b) cultured in the microchannel for 48 hours.

Low glucose (1.0 mg/ml) DMEM (Sigma, USA) is mixed 1:1 (v/v) with F-12 Ham nutrient mixture (Sigma, USA), the mixture is supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco, USA) and 1% (v/v) penicillin/streptomycin (Gibco, USA) to formulate the complete growth medium for culturing human vascular endothelial cell line CRL1730 isolated from umbilical vein. The endothelial cells are grown in the complete

medium in T-75 cell culture flask (NUNC, Denmark) at 37 °C with 5% CO₂. Confluent cells are trypsinized with 0.25% trypsin plus 0.05% EDTA (Gibco, USA). Harvested cells are concentrated by centrifugation and then resuspended in the growth medium at the concentration of about 1×10^7 cells/ml. The cell suspension is injected into the inlets of the microfluidic chip using a glass syringe (Hamilton, USA). The chips are then incubated in the cell culture incubator with medium replenishment every 24 hours until the cells in the microchannel reached almost full confluence for the experiments. Growth of the endothelial cells in the microchannels is monitored under the phase-contrast microscope (IX81, Olympus). Fig. 5.6(a) and Fig. 5.6(b) show the endothelial cells after initial seeding and after 48 hours culture in the microchannel with a well formed monolayer of cells.

In the experiments, 10 mM HEPES (Sigma, USA) is added into the culture medium to maintain the physiological pH value of the medium in the atmosphere environment. A heating platform is used to maintain the temperature of the microfluidic chip at 37°C for long-term observation under the microscope.

5.2 Endothelial cell dysfunction detection methods

5.2.1 Measurement of reactive oxygen species by H₂DCFDA

Figure 5.7: H₂DCFDA is hydrolyzed inside the cell, retained in the cytoplasm, and then oxidized by ROS, leading to the emission of green fluorescence.

The intracellular ROS level is measured using a cell-permeant fluorescent dye of 2', 7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) (D399, Invitrogen). The dye remains nonfluorescent until the acetate groups are removed by intracellular esterases, and oxidation occurs due to the presence of ROS (hydrogen peroxide and hydroxyl radicals) within the cells. Prior to the chemical and shear stress experiments, the cultured endothelial cells in the microfluidic chips are incubated in the culture medium with 10 μ M H₂DCFDA for 45 min to establish a stable intracellular level. The cells are then being exposed under several chemical and shear treatment conditions with the same concentration level of H₂DCFDA dissolved in culture medium. With the presence of ROS in the cells, the H₂DCFDA retained in the

cytoplasm is oxidized and becomes fluorescent as shown in Fig. 5.7. Therefore, the

(a)

(b)

Figure 5.8: The fluorescent images of endothelial cell treated with H_2DCFDA at (a) Low ROS level and (b) High ROS level.

fluorescence intensity of the cell is an index of the intracellular level of ROS.

The wavelengths of fluorescent excitation and emission of the H_2DCFDA dye, i.e. 475 nm and 525 nm, respectively. Immediately before measuring the fluorescence

intensity, the endothelial cells are washed with phosphate buffered saline (PBS) to remove the extracellular H₂DCFDA to achieve better signal/noise ratio. The fluorescent images are captured using an inverted fluorescence microscope (Axiovert S100, Carl Zeiss) equipped with a monochrome camera (RT3, SPOT imaging solutions) as shown in Fig. 5.8. For each chemical and shear treatment condition, 300 cells are utilized to measure the average fluorescence intensity. Each data point is normalized by the maximal intensity and expressed as a fraction of the maximum value.

5.2.2 Mitochondrial morphological measurement

Figure 5.9: Mitochondrial morphology detection by MitoTracker green.

The morphologies of the mitochondria can be investigated by staining the mitochondria inside the cells using a green-fluorescent dye (MitoTracker Green FM, Invitrogen). The MitoTracker Green FM is essentially nonfluorescent in an aqueous solution, and becomes fluorescent when it accumulates in an lipid environment in the mitochondria as shown in Fig. 5.9. Therefore, background fluorescence is negligible and the morphologies of the mitochondria can be visualized clearly.

Prior to the chemical and shear stress experiments, the cultured endothelial cells in the microfluidic chips are incubated in culture medium with a working concentration of 200 nM of MitoTracker dye for 45 min. After that, cells are washed with PBS to remove extracellular Mitotracker dye. With a stable intracellular level of MitoTraker dye, the cells are exposed under several chemical and shear treatment conditions. When excited at 490 nm, the fluorescence of the MitoTracker can be observed directly at the emission wavelength of 516 nm after the treatment. The morphologies of the mitochondria are studied via the captured fluorescent images using an inverted fluorescence microscope (Axiovert S100, Carl Zeiss) equipped with a monochrome

(a)

(b)

Figure 5.10: Fluorescent images of endothelial cells with: (a) Filamentous reticular mitochondrial networks, and (b) Mitochondrial fragmentation.

camera (RT, SPOT imaging solutions), as shown in Fig. 5.10 For each chemical and shear treatment condition, 300 cells are examined to determine the morphological changes of the mitochondria. Each data point is expressed as a mitochondrial fission ratio, which is the ratio of the number of cells with diffused mitochondria to the total number of cells evaluated.

5.2.3 Endothelial cell death measurement using yo pro-1 and propidium iodide

Figure 5.11: Chemical structures of yo pro-1 and propidium iodide.

The endothelial cell apoptosis and necrosis are quantified by using fluorescent dyes yo-pro-1 (Invitrogen, USA) and propidium iodide (PI) (Sigma, USA) as the first approach. The green-fluorescent yo pro-1 stain has found a niche in identifying apoptotic cells. Apoptotic cells become permeant to yo-pro-1, but remain impermeant to PI, a dead cell stain. Live cells are not stained with yo pro-1, allowing them to be used in subsequent experiments. The chemical structures of two the fluorescent dyes are shown in Fig. 5.11.

For this double-labeling technique, 50 μM of yo pro-1, diluted in phosphate buffer and 10 μM of PI are added in the culture medium. After treating the endothelial cells with different combinations of pulsatile flow profiles and glucose concentrations, the previous used culture medium is replaced with the medium containing these fluorescent dyes. Then, the microfluidic chip is incubated at 4°C for 30 min in a dark environment and subsequently washed with PBS to remove excess fluorescent dyes in the medium. The fluorescent images are captured by using fluorescence microscopy at an excitation

Figure 5.12 Measurement of cell apoptosis and necrosis by using yo pro-1 and propidium iodide: optical micrograph of apoptotic and necrotic fluorescing in green and red, respectively.

wavelength of 485 nm ($\lambda_{em} = 530$ nm) and 590 nm ($\lambda_{em} = 630$ nm) to detect apoptotic cells with yo pro-1 and necrotic cells with PI.

To validate the double-labeling fluorescent technique, the HUVECs are treated with 100 μ M etoposide (Sigma, USA), which is a well-known apoptotic inducer, as the positive control. For each treatment condition, 3 cell culture chambers are set as a parallel group. For each chamber, 10 observation sites containing at least 100 cells per site are

Figure 5.13: Statistical analysis on the rate of apoptosis and necrosis under different glucose concentrations for 12 h. Etoposide is used as the positive control to test the effect of double-labeling method.

counted (sample size of 1,000 cells). Apoptotic or necrotic ratios are normalized by determining the ratio between the number of labeled cells and the total number of cells.

With this double-labeling fluorescent method, apoptotic cells became permeant to yo pro-1 and fluoresced in green but remained impermeant to PI. Other dead cells are stained by PI and fluoresced in red as shown in Fig. 5.12. The auto-fluorescence intensity of the double-labeling system in normal culture medium and high glucose medium is firstly measured as the background level. In this series of experiments under static flow condition, a treatment with 100 μ M etoposide for 4 hours is used as the positive control of yo pro-1 fluorescent dye as it induces only HUVEC apoptosis without necrosis.

Etoposide is a widely-used anti-cancer agent, which forms a ternary complex with DNA and the topoisomerase II enzyme, preventing re-ligation of the DNA strands. It causes errors in DNA synthesis and promotes apoptosis of cancer cells [189]. Based on the statistical results as shown in Fig. 5.13, more than 50% of apoptotic cells but significantly low necrotic ratio (7-8%) for endothelial cells are observed with the etoposide treatment. This shows the specificity of yo pro-1 to distinguish apoptotic from necrotic cells. In the experiments, endothelial cells are exposed to 5, 10 and 20 mM glucose for 12 hours, respectively and the apoptotic and necrotic ratios are measured. The results show that increasing glucose concentration caused more endothelial cells to die by both apoptosis and necrosis. It is known that high glucose concentration can generate oxidative stress in endothelial cells through several pathways and consequently induce them to apoptosis. In addition, imbalanced concentration of glucose between two sides of the cell membrane impairs the membrane function and induces cell necrosis.

Although the double-labeling fluorescent technique is effective in detecting the endothelial cell apoptosis and necrosis, there exist several disadvantages. First, the operation process is complicated, requiring several preparation and processing steps, including storing the microfluidic chip at 4°C for 30 min during the dye staining stage. This brings undesired factors and influences to the endothelial cells. Most importantly, it will not allow a real-time study of the cell apoptotic process. The effect of different time durations of various treatment conditions cannot be accurate due to these post-treatment steps.

5.2.4 Endothelial cell apoptosis measurement via FRET biosensor

Caspase-3

Figure 5.14: Principle of a living cell biosensor which detects caspase-3 activation using FRET: (a) Living HUVEC-C3 with a 535 nm green fluorescent emission and (b) Dead HUVEC-C3 with cleaved linker peptide and a 480 nm blue fluorescent emission.

In order to detect living HUVEC cells in the microfluidic chip, we have developed HUVEC-C3 cell line transfected with the FRET biosensor caspase-3 [190, 191]. The biosensor caspase-3 is a recombinant substrate protein for caspase-3 and consists of three parts: a donor cyan fluorescent protein (CFP), a 16 amino acid peptide linker containing the caspase-3 cleavage sequence Asp-Glu-Val-Asp (DEVD), and an acceptor yellow fluorescent protein (YFP) as shown in Fig. 5.14. When HUVEC-C3 cells are alive, using an excitation light with wavelength of 430 nm, the CFP is excited and its fluorescent

emission energy is transferred to YFP inside the cells, resulting in a green fluorescent emission with $\lambda_{em} = 530$ nm. When caspase-3 is activated as the cell is undergoing apoptosis, the linker peptide between CFP and YFP is cleaved by the caspase-3, abolishing the FRET effect and resulting in a change of emission light from green color to blue with $\lambda_{em} = 480$ nm. To measure the *in vivo* FRET effect, the microfluidic chip with HUVEC-C3 is excited using a band-pass filter (430 ± 10 nm). The emission images of YFP (530 ± 10 nm) and CFP (480 ± 10 nm) are recorded using a computer-controlled cooled CCD camera (Princeton Instruments, Inc., NJ). The digital fluorescence images are then processed using the MetaMorph software (Universal Imaging Co., PA), to obtain the Y/C emission ratio which indicates the caspase-3 activation in the living apoptotic cells. To validate the FRET sensor method, the HUVCE-C3 are treated with 500 nmol/L Taxol (Sigma, USA) or exposed to UV-light for 3 min as the positive control. For each chamber, 10 observation site containing at least 100 cells are counted. The final fluorescent intensity results from the percentage of the CFP cell number among the whole observed cell group.

To evaluate the influence of the combination of different pulsatile shear stresses and glucose concentrations in real-time, HUVEC-C3 cells constitutively produce a caspase-3 sensor capable of detecting caspase activation in living endothelial cells is used. The VEC-C3 cells are induced to apoptosis by 3-min UV-irradiation or incubation with 500 nM Taxol. The UV-mediated apoptosis is a highly complex process in which different molecular pathways are involved, including DNA damage, activation of the tumor suppressor gene p53, triggering of cell death receptors either directly by UV or by autocrine release of death ligands, mitochondrial damage and cytochrome C release [192].

The anti-cancer agent, Taxol, stabilizes microtubules leading to G2/M cell cycle arrest and subsequently apoptotic cell death [193]. The CFP image (colored blue) and the YFP image (colored green) of the same area of cells are merged together and displayed as shown in Fig. 5.15(a). Fig. 5.15(b) shows the increased apoptotic rate at 24 hours after the treatment of UV-radiation and Taxol. UV-irradiation and Taxol are both well-known apoptotic inducers, which induced 24% and 9% cell apoptosis as measured by the sensor caspase-3 cells. The statistical results show that the sensor caspase-3 cells are reliable in detecting cell apoptosis. In addition, the exposure of 20 mM glucose for 24 hours also induced a slight increase in cell apoptosis but not significant under the static flow condition.

Figure 5.15: Detection of caspase-3 activation in living apoptotic cells based on FRET effect. (a) Merged FRET image of HUVEC-C3 with live cells appeared in green color and apoptotic cells in blue color and (b) Statistical analysis of apoptotic rate after 24 h of 3 min UV-exposure, 500 nM of Taxol, and 20 mM of glucose treatments.

5.3 Endothelial cell dysfunction detection

5.3.1 Measurement of reactive oxygen species overproduction

Four different shear treatment conditions are selected to investigate the shear-induced responses on cultured endothelial cells in the microchannel: (1) static condition (without a fluidic flow), (2) constant shear stress of 15 dyne cm^{-2} with a flow rate of $8.46 \mu\text{L s}^{-1}$, (3) normal physiological pulsatile shear stress of 15 dyne cm^{-2} (mean) with a frequency of 70 bpm, and (4) physiological pulsatile shear stress of 30 dyne cm^{-2} (mean) with a frequency of 140 bpm to mimic the condition under exhaustive exercise. The temporal responses of the endothelial cells in ROS production level under different shear treatment conditions are shown in Fig. 5.16. The static condition serves as a negative control of the experiment and it can be seen that the fluorescence intensity increased when the dye molecules stained the cells for longer than 15 – 30 min. To exclude this intensity increment, the normalized fluorescence intensities for the other three shear treatment conditions are subtracted from that of the negative control. The intracellular ROS when the endothelial cells are being exposed to pulsatile shear stress of 30 dyne cm^{-2} for different time periods is increased nearly 4-fold in the first 60 min. Prolonged exposure of the endothelial cells to the shear flow up to 2 hours resulted in a sustained elevation in ROS levels, which is same with what is previously [139]. By comparing the ROS levels in the four shears treatment conditions after duration of 60 min. It can be seen that there is no significant increase in the ROS level between the case with constant shear stress of 30 dyne cm^{-2} and the negative control which has no shear stress as the culture medium is maintained in a static condition in the microchannel (0.36/0.34). In contrast, the increment is more substantial when the endothelial cells are being exposed to

Figure 5.16: Time-dependent responses of the ROS level in endothelial cells under different shear stress profiles.

physiological pulsatile shear stress conditions. Compare to the static case, the ROS level is increased by 1.7 fold ($0.56/0.34$) and 2.2 fold ($0.76/0.34$) at pulsatile shear stress level of 15 and 30 dyne cm^{-2} , respectively. This shows that it is necessary to investigate the effect of shear stress on the endothelial cells under a physiological condition which can represent the pulsatile patten of the blood flow in the artery. In addition, reports stating that pulsatile shear stress is critical to maintain the functionalities of endothelial cells in the artery have been presented [194, 195]. Therefore, it is more realistic to expose the endothelial cells using pulsatile shear stress under different chemical conditions in the experiments.

For normal people, the concentration of glucose in the blood is ranging from 4 to 6 mM. On the other hand, for diabetes patients, the concentration of glucose in the

blood is above 6 mM and may be up to 20 mM. In these experiments, 10 mM of glucose is added to mimic the plasma condition of diabetes patient and the ROS level of the endothelial cells are monitored under normal physiological pulsatile shear stress condition or under exhaustive exercise. The experimental results for the normal physiological pulsatile shear stress condition is shown in Fig. 5.17. When the endothelial cells are being exposed to 10 mM glucose under the physiological pulsatile shear stress of 15 dyne cm^{-2} , the ROS level is gradually increased in the first 60 min and stabilized afterward. By comparing the ROS level at 60 min time frame between the cells treated with or without 10 mM glucose, but both under a shear stress of 15 dyne cm^{-2} , the ROS level is elevated by 45% ($0.81/0.56$) (Graph in Fig. 5.16). It can be concluded that high glucose induces the formation of intracellular ROS level.

Figure 5.18 shows the experimental results of endothelial cells being exposed to the physiological pulsatile shear stress of 30 dyne cm^{-2} (exhaustive exercise). Similar to the ROS elevation profile seen under the pulsatile shear stress of 15 dyne cm^{-2} , the ROS level is also gradually increased in the first 60 min and stabilized afterward. By comparing the ROS level at 60 min time frame between the cells treated with or without 10 mM glucose, but both under the high level of share stress of 30 dyne cm^{-2} , the ROS level is elevated by 22% ($0.93/0.76$) (Graph in Fig. 5.17). By comparing the ROS level at 60-min time frame between the one with pulsatile shear stress of 15 dyne cm^{-2} and the one with pulsatile shear stress of 30 dyne cm^{-2} , the ROS level is elevated by 15% ($0.93/0.81$) (Graphs in Fig. 10 and 11). It can be concluded that high glucose level under exhaustive exercise condition can

substantially increase the intracellular ROS level.

Fig 5.17 The ROS level in endothelial cells being exposed to 10 mM glucose with pulsatile shear stress of 15 dyne cm^{-2} .

(a)

(b)

Figure 5.18 The ROS level in endothelial cells being exposed to 10 mM glucose with pulsatile shear stress of 30 dyne cm^{-2} .

5.3.2 Measurement of mitochondrial morphological change

(a)

(b)

Figure 5.19: Mitochondrial morphology of endothelial cells under different shear treatment conditions for 3 hours.

The mitochondrial morphology of endothelial cells being exposed under different shear treatment conditions are investigated using MitoTracker staining and the fluorescent images are shown in Fig. 5.19. The endothelial cells under static condition (negative control) are mostly tubular with the appearance of filamentous reticular networks. Only 2% of the cells have shorter and smaller mitochondria, which indicate mitochondria fragmentation. When the endothelial cells are exposed to a physiological pulsatile shear stress, some of the cells showed mitochondrial morphological changes. The mitochondrial fission ratio of the endothelial cells under these conditions for the first 3 hours are shown in Fig. 5.19. The level of endothelial cell mitochondrial fragmentation after being exposed to pulsatile shear stress of 15 and 30 dyne cm⁻² for 3 hours are measured as 6.8% and 7.6%, respectively. The results show that endothelial cells would remain their viability and function under normal physiological pulsatile shear stress or during exhaustive exercise.

To investigate the mitochondrial morphological changes of endothelial cells, 20 mM of glucose is added and the endothelial cells are being exposed under different shear treatment conditions for 3 hours. The fluorescent images of the endothelial cells after being exposed for 3 hours and the mitochondrial fission ratios monitored for the first 3 hours are shown in Fig. 5.20. For endothelial cells treated with 20 mM glucose for 3 hours, mitochondrial fragmentation is prevalent with a mitochondrial fission ratio of 20%. When the endothelial cells are being exposed with 20 mM glucose plus the pulsatile shear stress of 15 or 30 dyne cm⁻², the mitochondrial fission ratio is further elevated from 20% to 25% and 26%, respectively. The results show that the chemical treatment (i.e. high concentration of glucose) produced a more dominant

(a)

(b)

Figure 5.20: Mitochondrial morphology of endothelial cells with 20 mM glucose under different shear treatment conditions.

effect in promoting mitochondrial fission than the physical treatment (i.e. pulsatile shear stress), especially in such an extreme case of 20 mM of glucose concentration.

5.3.3 Measurement of endothelial cell apoptosis and necrosis

By using the double-labeling fluorescent method, the apoptotic and necrotic state of HUVEC under different combinations of pulsatile shear stresses and glucose concentrations are investigated. First, the endothelial cells are exposed to 5, 10 and 20 mM glucose concentration under a flow with shear stress level of 30 dyne cm⁻² for 2 hours and subsequently shear stress 15 dyne cm⁻² for 10 hours. For diabetes patients, a glucose concentration in blood of 10 mM is a common condition without any medicine control, while 20 mM is an extremely high level. Fig. 5.21(a) shows the statistical results of the effect of glucose concentration on endothelial cell apoptosis and necrosis with the presence of pulsatile flow profile. At the normal blood glucose level (5 mM) the intense pulsatile shear stress induced insignificant cell apoptosis (2.5%) and necrosis (1%) simultaneously. When the glucose concentration increases to 10 mM, a near 3-fold increment in the cell death ratio is detected (7% for apoptosis and 3% for necrosis). Both apoptotic (12%) and necrotic (5%) rates are further elevated when the glucose concentration increases to 20 mM. By comparing there results with these of the static condition shown in Fig. 5.21(b), it can be concluded that high glucose concentration has more apparent effect on triggering apoptosis and necrosis in HUVEC, especially under exhaustive pulsatile flow profile.

To further investigate the impact of pulsatile shear stress on inducing cell death, the glucose concentration is maintained at a high level of 20 mM, while three different pulsatile flow profiles are tested: (1) a normal pulsatile with a shear stress of 15 dyne cm⁻² for 12 hours is used to represent the shear force in the resting condition, (2) an exhaustive pulsatile flow condition of shear stress of 30 dyne cm⁻² for 1 hour and

followed with a normal shear stress 15 dyne cm^{-2} for 11 hours, and (3) an exhaustive pulsatile flow with a shear stress of 30 dyne cm^{-2} for 2 hours and followed with a normal shear stress of 15 dyne cm^{-2} for 10 hours. The statistical results in Fig. 5.21(b), show that under the high glucose concentration condition, the pulsatile shear stress of the normal physiological level (15 dyne cm^{-2}) generated 14% of cell death rate, which is higher than the 11% death rate generated by the same high glucose in the static conditions). The death rates are further increased to 17.5% when a more exhaustive shear stress of 30 dyne cm^{-2} is applied to cells for 2 hours. These results show that it is more accurate and realistic to expose the endothelial cells to pulsatile shear stress during the investigation of the endothelial cell response under different chemical conditions because it can better mimic the in vivo situation in the blood vessels. This means that for diabetes patients with a high glucose concentration in the blood vessels, an exhaustive blood flow will induce higher death rate for the endothelial cells, which will further damage the function of the blood vessels.

Figure 5.21: Measurement of cell apoptosis and necrosis by using yo pro-1 and propidium iodide, (a) Statistical analysis on the rate of apoptosis and necrosis under different glucose concentrations with a flow profile of 2 h SS 30 (30 dyne cm^{-2}) and 10 h SS15 (15 dyne cm^{-2}) and (b) Statistical analysis on the rate of apoptosis and necrosis different flow profiles conditions with 20 mM glucose. Sample size: 1,000 cells.

Fig 5.22 FRET-based detection of caspase-3 activation in living apoptotic cells shows the results of statistical measurements of the percentage of apoptotic cells using caspase-3 activation under different glucose concentrations and different flow profile conditions. Sample size: 1,000 cells.

Similar to the investigations for double-labeling technique, the FRET changes in statistical results are shown in Fig. 5.22. First, the percentage of apoptotic under different glucose concentrations (5, 10 and 20 mM) either under a 2 hours exhaustive pulsatile flow with a shear stress 30 dyne cm^{-2} and 10 hours normal pulsatile flow with a shear stress of 15 dyne cm^{-2} are compared and analyzed. The YFP/CFP emission ratio, which indicated the caspase-3 activation in living apoptotic cells apparently decreases with the elevation of glucose concentration as shown in Fig. 5.23. To observe a more obvious FRET change, the final YFP/CFP emission ratio is detected 48 hours after the treatment to provide more time for caspase activation. By increasing the glucose concentration from the normal physiological level of 5 mM to 10 mM, a nearly 2-fold increment of the apoptotic rate is observed (from 6.5% to 11%) under the shear stress of 2 h-SS30 and 10 h-SS 15. Under the highest glucose concentration of 20 mM, about 12.5% of the endothelial cells underwent a caspase-3 related apoptotic cell death, which could significantly affect the maintenance of the normal blood vessel function [196]. Secondly, the apoptotic rate of endothelial cells under the same glucose concentrations of 20 mM with different pulsatile flow profiles were compared and analyzed. The FRET images are illustrated in Fig. 5.24. It can be seen that cell apoptosis via the caspase-3 pathway is minimal under a normal pulsatile condition of shear stress 15 dyne cm^{-2} for 12 hours. When a longer period of the exhaustive pulsatile shear stress of 30 dyne cm^{-2} is applied to the cells in the present of 20 mM glucose, the measured apoptotic rate is significantly increased from 4% to 12.5%. Adding one hour of exhaustive pulsatile shear stress nearly doubled the apoptotic rates of the endothelial cells in all three glucose concentrations. These results show the potential damaging effect of excessive mechanical force to

endothelial cells, especially in combination with high concentration of glucose at 20 mM. For the diabetes patients with a high glucose concentration in the blood vessels, it is quite danger to have an exhaustive blood flow lasting for long time, which will induce high rate of apoptosis of the endothelial cells. As the exhaustive blood flow time become longer, it is more dangerous to the diabetes patients. 2 hours exhaustive blood flow under high glucose concentration maybe induces quite serious damage to the blood vessels

To compare the two cell death detection methods and obtain a deeper understanding of the pulsatile shear stress and the high glucose concentration effect, a comparison of the time-dependent responses of the ROS level, mitochondrial damage, caspase-3 activation and cell apoptotic rate under the same condition of 20 mM glucose concentration and 15 dyne cm^{-2} shear stress is carried out, and there results are shown in Fig. 5.25. The occurrence of these primary corresponding changes is in sequence. On one hand, when the endothelial cells are exposed to the pulsatile shear stress and high glucose concentration, the ROS level increased immediately within 30 min (from 20% to 90%) and reached the peak in 60 min (100%). Maintaining this severe stress treatment induce further mitochondrial damage, which manifested as a morphological change to mitochondrial fission within 4 hours (from 2% to 25%). If the high glucose concentration and pulsatile shear stress microenvironment continued until the endothelial cells lose their own self-repairing function, the massive apoptosis is induced. The yo-pro-1 and PI double-labeling fluorescent technique can detect all apoptotic and necrotic types. On the other hand, the biosensor C3 probe method can detect caspase-3 dependent apoptosis more precisely in real-time. Since caspase-3 activation is the main pathway of the pulsatile shear stress and high glucose concentration induced cell death, the two

measurements are comparable as shown in Fig. 5.25. The pulsatile shear stress condition-dependent cell measured by the sensor C3 probe is more accurate due to its real-time observation as well as avoiding other disturbing possibilities. In addition, it is observed that the time-dependent increment trend and sequence are consistent among the ROS level, the mitochondrial damage rate and the cell apoptotic rate.

(a)

(b)

(c)

Figure 5.23: FRET changes in response to caspase-3 activation with different glucose concentrations (a) 5 mM (b) 10 mM and (c) 20 mM under shear stress 30 dyne cm^{-2} 2 h plus shear stress 15 dyne cm^{-2} 10 h.

Figure 5.24: FRET changes in response to caspase-3 activation under different conditions

(a) 5 mM glucose under shear stress of 15 dyne cm^{-2} for 12 h, (b) 20 mM glucose under shear stress of 15 dyne cm^{-2} for 12 h (c) 20 mM glucose under shear stress of 30 dyne cm^{-2} for 1 h plus shear stress of 15 dyne cm^{-2} for 11 h and (d) 20 mM glucose under shear stress of 30 dyne cm^{-2} for 2 h plus shear stress of 15 dyne cm^{-2} for 12 h.

Figure 5.25: Time-dependent responses of: (a) ROS level, (b) Mitochondrial damage and (c) Caspase-3 activation and cell apoptotic rate are measured by yo pro-1 with glucose concentration of 20 mM and under different flow conditions.

5.3.4 Inhibition of endothelial cell dysfunction using antioxidants

Figure 5.26: Effect of propyl gallate (PG) and butylated hydroxyanisole (BHA) on shear-induced increase of ROS production in endothelial cells.

An antioxidant is capable of inhibiting the oxidation of other molecules, which is a chemical reaction that transfers electrons or hydrogen from a substance to an oxidizing agent. Antioxidants are used in this study to test their protective effect on oxidative stress-induced cell apoptosis. The endothelial cells are pre-treated for 1 hour with different kinds of antioxidants, including 100 μ M butylated hydroxyanisole (BHA), 1 mM L-glutathione reduced (GSH), 20 μ M propyl gallate (PG) and 1 mM N-acetylcysteine (NAC), respectively. After the pre-treatment, cells are further treated with different combinations of pulsatile flow profiles and glucose concentrations. Finally, the

apoptotic rates are measured and quantified.

To evaluate the shear-induced ROS production, two antioxidants are examined, i.e. propyl gallate (PG) and butylated hydroxyanisole (BHA). PG is an antioxidant which inhibits the oxidation by hydrogen peroxide and oxygen free radicals. On the other hand, BHA is an antioxidant which stabilizes free radicals and acts as a free radical scavenger. In the experiments, the endothelial cells are pretreated with either one of the antioxidants at 20 μM for 30 min. Subsequently, the endothelial cells are being exposed to the pulsatile shear stress of 30 dyne cm^{-2} for one hour, and the ROS levels are simultaneously measured, as shown in Fig. 5.26. The ROS level is increased by 4-fold without the addition of antioxidants. The ROS level is significantly reduced by PG, but BHA did not show any obvious oxidation protective effect.

To determine the importance of the ROS production and the oxidative stress to the endothelial dysfunction, the effect of four antioxidants are examined, 100 μM butylated hydroxyanisole (BHA), 1 mM L-glutathione reduced (GSH), 20 μM propyl gallate (PG) and 1 mM N-acetylcysteine (NAC). In the experiments, the endothelial cells are pre-treated with each of the antioxidants for 1 hour and subsequently, being exposed to the exhaustive pulsatile shear stress of 30 dyne cm^{-2} for 2 hours and followed with a normal shear stress of 15 dyne cm^{-2} for 10 hours, as shown in Fig. 5.27. To evaluate the effect of these anti-oxidants, the YFP/CFP emission ratio is detected immediately after the treatment. The results show in Fig 5.27 indicate that PG but not the other three antioxidants, can prevent Bax translocation to mitochondria and cytochrome c release, which are the two up-stream events of caspase-3 dependent cell apoptosis [197].

Figure 5.27: Effect of antioxidant drugs on high glucose and shear stress induced cell apoptosis.

5.4 Summary

This chapter focuses on the design of a hemodynamic optofluidic chip, which is used to better understand how hyperglycemia induces endothelial cell dysfunction under diabetic conditions.

The production of reactive oxygen species (ROS) in endothelial cells grown in a microchannel under different physical (shear stress) and chemical (glucose concentration) conditions is studied. Additionally, the system also included a caspase-3 based fluorescence resonance energy transfer (FRET) biosensor cell line.

The hemodynamic optofluidic system is demonstrated to be a fully biocompatible and highly controllable microfluidic platform in which cells can be cultured with a controlled perfusion of culture medium. The designed Lab-on-a-chip system mimics the flow rate of blood in the artery at the resting condition or after an exhaustive exercise. The measurement results show that pulsatile shear stress is an essential element to mimic the physiological conditions in the blood vessel

The intracellular ROS level is determined using real-time fluorescence microscopy with the measurement of the oxidation of 2', 7'-dichlorodihydrofluorescein diacetate by hydroxyl radicals or hydrogen peroxide molecules. The elevated ROS level in the mitochondria led to morphological changes of mitochondria from filamentous reticular networks to diffused and short fragments. The shear-induced cellular responses of endothelial cells under glucose concentrations of 10 mM or 20 mM are realized and studied by mimicking the physiological pulsatile flow profile in blood vessel using the developed hemodynamic Lab-on-a-chip system.

A double-labeling fluorescent technique (yo-pro-1 and propidium iodide) is used to validate the findings revealed by the FRET-based caspase sensor. The results show high rates of apoptosis and necrosis of endothelial cells when high glucose concentration is applied in our hemodynamic microfluidic chip combined together with an exhaustive pulsatile flow profile. The two apoptosis detection techniques (fluorescent method and FRET biosensor) which has been used in our studies are found to be comparable; however, the FRET biosensor offers more advantages such as real-time observation and a more convenient operation which generates more accurate and reliable data.

The target of this study is to develop an endothelial cell dysfunction analysis method, which combines the microfluidic chip system with different biosensors. The findings of our study may provide new insights on how glucose causes endothelial cell dysfunction that is the major cause of diabetes-derived complications.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

Three different innovative types of optofluidic chips for living cell culture and bacteriophage detection have been designed in this thesis. First, a reconfigurable optofluidic grating is designed and fabricated using multiphase droplets. Second, a dedicated microdroplet optofluidic chip is used for detecting λ bacteriophages and M13 bacteriophages, which are co-cultured with the host cell *E. coli* and then divided into large number of droplets. Third, a novel endothelial cell apoptosis detection method, which combines a caspase-3 based FRET biosensor cell line using a optofluidic chip has been successfully developed and demonstrated. The major conclusions of this Ph.D project are as follows

An optofluidic grating using multiphase microfluidic droplets is designed and demonstrated. It employs only one row of droplets and optical illumination from the top. It has a simple structure which can be easily fabricated, a high sensitivity, improved tuning speed, and exhibits high uniformity and high stability in its operation.

(i) Using a simple T-junction geometry, PBS buffer droplets are generated in immersion oil. A one-dimension grating along the channel is formed by using the subsequent droplet flow stream and microdroplet self-patterning. The size of the droplet can be controlled over a broad range by the equation that describes the relationship

between the droplet size and flow rates of two immiscible phases. These optofluidic-based grating is intrinsically well suited for dynamic and reversible control of the optical properties.

(ii) The diffraction pattern of the droplet grating is deduced and analyzed, as predicted by theory, the measured data show it is a combination of the Fraunhofer diffraction of single circular aperture and the interference of multiple slits. For the monochromatic incidence, the diffraction patterns corresponding to the grating periods of $d = 47.3, 28.0$ and $23.1 \mu\text{m}$ is captured and the patterns exhibit bright rings overlapped on bright stripes as predicted by the theoretical analysis. For the white incident light, the increase of n_{droplet} from 1.439 to 1.447 causes the transmitted color to vary from magenta to red is also detected.

(iii) The whole optofluidic system can be used as an optofluidic refractometer by analyzing the diffraction patterns. A variation of n_{droplet} from 1.341 to 1.357 corresponded to a change over the whole intensity range. Therefore, the detection limit is approximately 6.3×10^{-5} RIU. The optofluidic system can also be used as a color filter. With the change of the refractive index of the CaCl_2 solution, the central wavelength of the filtered color is shifted at a rate of $\sim 50 \text{ nm/RIU}$.

A droplet optofluidic system to detect bacteriophages via measuring the growth of the host cell infected by the bacteriophages in a microdroplet carrier has been designed and demonstrated. The system provides a real-time, label-free and high sensitivity quantification of bacteriophage through the measurement of host cell growth with high accuracy and stability.

- (i) The bacteriophage sample is first mixed with host cell *E. coli* culture medium and then divided into a large number of droplets. After an incubation period, the host cell growth in the bacteriophage-contained microdroplets should be obviously different from the microdroplets containing only host cells. The final concentration of the host cell affects the scattering pattern of the microdroplet, which can be used to detect the existence of the bacteriophage.
- (ii) The mean power frequency value of the single microdroplet scattering pattern increases with the increment of the concentration of *E. coli* in the microdroplet. Based on the standard curve, the number of *E. coli* can be calculated when we get the scattering pattern of a microdroplet containing unknown concentration of host cells. The concentration varies of *E. coli* cultured individually or co-cultured with λ phage and M13 phage in the droplet comparing with in flask is detected using this optofluidic chip.
- (iii) Water sample containing different concentration bacteriophage is measured to verify the detection ability of the optofluidic. The optofluidic system can detect the water sample with known bacteriophage concentration of $C_{bacteriophage} = 10^3$ pfu/ml and $C_{bacteriophage} = 10^4$ pfu/ml. The optofluidic system can detect lower concentration of bacteriophage by increasing the microdroplet number of each group.

The shear-induced cellular responses of endothelial cells under different glucose concentrations are realized by mimicking the physiological pulsatile flow profile in blood vessel. The apoptosis rate matches well with the ROS overproduction and mitochondria damage rate and the study will further uncover the role of diabetes as a contributor to endothelial dysfunction and cardiovascular diseases.

- (i) The optofluidic-based hemodynamic chip is designed and fabricated. The chip consists of different layers which contain the concentration gradient network with three branches of microchannels and series cell culture sites to perform a series of time course experiments. A pulsation free precision pump is used to control flow and the flow profile of the pump is configured to mimic the blood flow in a blood vessel.
- (ii) The intracellular ROS level is determined using real-time fluorescence microscopy with the measurement of the oxidation of 2', 7'-dichlorodihydrofluorescein diacetate by hydroxyl radicals or hydrogen peroxide molecules. The elevated ROS level in the mitochondria leads to the morphological changes of mitochondria from filamentous reticular networks to diffused and short fragments. The shear-induced cellular responses of endothelial cells under glucose concentrations of 10 mM or 20 mM are realized and studied by mimicking the physiological pulsatile flow profile in blood vessel using the developed hemodynamic optofluidic system.
- (iii) The activation of the FRET biosensor confirms the endothelial cell apoptosis induced by the abnormal pulsatile shear stress and high glucose concentration is through caspase-3 pathway. A 12% apoptotic rate (nearly 4-fold as compared with the static condition) is observed when the endothelial cells are exposed to a high glucose concentration of 20 mM under 2 hours exhaustive pulsatile shear stress of 30 dyne cm^{-2} which are then followed by another 10 hours of normal pulsatile shear stress of 15 dyne cm^{-2} .

In conclusion, optofluidic technologies enable the incorporation of several functionalities which facilitate the culture and detection of living cell with higher stability, efficiency and ease of measurement in a more controllable environment.

Another great advantage of the microfluidic network is that the living cell can be easily manipulated and settled down at measurement area. The microdroplet-based carrier and microreactor enable living cells to be compartmentalized and characterized by measuring the optical absorbance, fluorescence intensity or scattering signal of the droplets. The droplet techniques have the benefits of microscale with decreased diffusion distance, faster mixing, and laminar flow. Such continuous systems can also include a large number of microreactors to allow parallel processing while keeping each reactor independent and isolated. These unique properties have enabled a wide array of biochemical diagnostic assay to be performed using droplet-based optofluidic systems. To further improve the development of optofluidic chips for living cell culture and measurement, several future works with relevant recommendations for future work are described in the following section.

6.2 Recommendations

The recommendations for future research are summarized as follows

- a) Based on the current study of the microfluidic droplet grating, more work is scheduled in the research plan. For the next stage, further applications using the droplet grating for chemical and biological analysis will be carried out. The two immiscible phases will be displaced by more complicated chemical and biological solutions. Furthermore, biomolecules or single cells will be encapsulated inside the microdroplets. Using the detection capability of the grating system, more information can be collected for chemical and biological studies.
- b) For the droplet-based optofluidic system, the most important aspect is to improve the working efficiency and detection limitation. The goal is to develop a system that is capable of detect bacteriophages with concentrations < 1 pfu/ml within 2-4 hours with high accuracy and limited human intervention. Since our reported optofluidic microsystem exhibits high sensitivity and specificity, it is expected to lay a foundation for the next generation of water monitoring instrumentation. The system can first be applied to healthcare and food sectors where drink water is vital. Eventually this most advanced drinking water system can be put to work at each family or household.
- c) The hemodynamic optofluidic chip system has successfully mimicked the microenvironment of the human blood vessel. However, a single layer of cultured endothelial cells is still different from the real conditions. The interactions between the endothelial cells and other blood vessel cells have not been explored. To solve this question, a cell co-culture microchip is suggest for future work.

Furthermore, the 2-dimensional (2D) endothelial cell distribution is not the best model. A totally 3-dimensional (3D) microchannel with a circular growth of endothelial cell is the most similar structure. For this purpose, more work is needed on microchannel design and fabrication techniques. Developing a viable 3D cell culture method is also necessary. Finally, considering only glucose concentrations is not enough for a holistic understanding of the health state of a diabetes patient, although it is the most significant factor. Therefore, it will be more accurate to use real blood sample in future experimental designs. However, in order to meet this new and much more requirement, the microfluidic system will need to provide more functions such as blood cell filtration and blood viscosity analysis. The detection sensor also requires further development for providing additional information on the complex interactions and the apoptosis pathway inside the endothelial cell.

AUTHOR'S PUBLICATIONS

Journal Papers

1. J. Q. Yu, Y. Yang, A. Q. Liu, L. K. Chin and X. M. Zhang, "Microfluidic droplet grating for reconfigurable optical diffraction," **Optics Letters**, Vol. 35, No. 11, pp. 1890-1892, 2010.
2. L. K. Chin, J. Q. Yu, Y. Fu, T. Yu, A. Q. Liu and K. Q. Luo , "Production of reactive oxygen species in endothelial cells under different pulsatile shear stresses and glucose concentrations," **Lab on a Chip**, Vol. 11, No. 11, pp. 1856-1863, 2011.
3. Z. G. Li, K. Ando, J. Q. Yu, A. Q. Liu, J. B. Zhang and C. D. Ohl, "Fast on-demand droplet fusion using transient cavitation bubbles," **Lab on a Chip**, Vol, 11, No. 11, pp 1879-1885, 2011.
4. J. Q. Yu, X. F. Liu, L. K. Chin, A. Q. Liu and K. Q. Luo, "Study of endothelial cell apoptosis using fluorescence resonance energy transfer (FRET) biosensor cell line with hemodynamic microfluidic chip system," **Lab on a Chip**, DOI: 10.1039/c3lc50105a, 2013.
5. J. Q. Yu, W. Huang, L. K. Chin, L. Lei, Y. J. Zheng, W. Ser and A. Q. Liu, "Rapid λ -Bacteriophage Detection via Co-culture of Host Cell *Escherichia coli* by Droplet-optofluidic System," **Lab on a Chip**, 2014 (accepted).

Conference Papers

6. J. Q. Yu, L. K. Chin, Y. Yang, Sock Hoon Ng, Peng Huat Yap and A. Q. Liu, "Droplet optical diffraction grating array," μ TAS2009 (13th International Conference on Miniaturized Systems for Chemistry and Life Sciences), Jeju Island,

South Korea.

7. J. Q. Yu, L. K. Chin, Y. Chen, G. J. Zhang, G. Q. Lo, T. C. Ayi, P. H. Yap D. L. Kwong and A. Q. Liu, "Microfluidic droplet-based liquid-liquid extraction for fluorescence-indicated mass transfer," μ TAS2010 (14th International Conference on Miniaturized Systems for Chemistry and Life Sciences), Groningen, Netherlands.
8. J. Q. Yu, L. K. Chin, A. Q. Liu and K. Q. Luo, "Pulsatile shear stress and high glucose concentrations induced cell death in endothelial cells," μ TAS2011 (15th International Conference on Miniaturized Systems for Chemistry and Life Sciences), Seattle, USA.
9. J. Q. Yu, W. Huang, L. K. Chin and A. Q. Liu, "Rapid bacteriophage detection via host cell amplification in a droplet-based optofluidic system," μ TAS2012 (16th International Conference on Miniaturized Systems for Chemistry and Life Sciences), Okinawa, Japan.
10. J. Q. Yu, W. Huang, L.K. Chin, L. Lei, Y. J. Zheng, W. Ser and A. Q. Liu, "Rapid λ bacteriophage detection via co-culture of host cell *Escherichia Coli* droplet optofluidic system." μ TAS2013 (17th International Conference on Miniaturized Systems for Chemistry and Life Sciences), Freiburg, Germany.

BIBLIOGRAPHY

1. H. Schmidt and A. R. Hawkins, *The photonic integration of non-solid media using optofluidics*. Nature Photonics, 2011. **5**(10): p. 598-604.
2. D. Psaltis, S. R. Quake, and C.H. Yang, *Developing optofluidic technology through the fusion of microfluidics and optics*. Nature, 2006. **442**(7101): p. 381-386.
3. X. L. Mao, J. R. Waldeisen, B. K. Juluri and T. J. Huang, *Hydrodynamically tunable optofluidic cylindrical microlens*. Lab on a Chip, 2007. **7**(10): p. 1303-1308.
4. H. Y. Zhu, I. M. White, J. D. Suter, P. S. Dale, X. Fan, *Analysis of biomolecule detection with optofluidic ring resonator sensors*. Optics Express, 2007. **15**(15): p. 9139-9146.
5. H. Schmidt and A. R. Hawkins, *Optofluidic waveguides: I. Concepts and implementations*. Microfluidics and Nanofluidics, 2008. **4**(1-2): p. 3-16.
6. X. D. Fan and I. M. White, *Optofluidic microsystems for chemical and biological analysis*. Nature Photonics, 2011. **5**(10): p. 591-597.
7. S. K. Sia and G. M. Whitesides, *Microfluidic devices fabricated in poly(dimethylsiloxane) for biological studies*. Electrophoresis, 2003. **24**(21): p. 3563-3576.
8. T. Vilkner, D. Janasek and A. Manz, *Micro total analysis systems. Recent developments*. Analytical Chemistry, 2004. **76**(12): p. 3373-3385.
9. J. El-Ali, P. K. Sorger, and K. F. Jensen, *Cells on chips*. Nature, 2006. **442**(7101): p. 403-411.
10. X. Hong, X. F. Gao, and L. Jiang, *Application of superhydrophobic surface with high adhesive force in no lost transport of superparamagnetic microdroplet*. Journal of the American Chemical Society, 2007. **129**(6): p. 1478-+.
11. R. Tewhey, J. B. Warner, M. Nakano, B. Libby, M. Medkova, et al., *Microdroplet-based PCR enrichment for large-scale targeted sequencing*. Nature Biotechnology, 2009. **27**(11): p. 1025-U94.

12. P. A. Auroux, D. Iossifidis, D. R. Reyes, A. Manz A, *Micro total analysis systems. 2. Analytical standard operations and applications*. Analytical Chemistry, 2002. **74**(12): p. 2637-2652.
13. A. J. deMello, *Control and detection of chemical reactions in microfluidic systems*. Nature, 2006. **442**(7101): p. 394-402.
14. A. Gunthe and K. F. Jensen, *Multiphase microfluidics: from flow characteristics to chemical and materials synthesis*. Lab on a Chip, 2006. **6**(12): p. 1487-1503.
15. D. R. Reyes, D. Iossifidis, P. A. Auroux and A. Manz, *Micro total analysis systems. 1. Introduction, theory, and technology*. Analytical Chemistry, 2002. **74**(12): p. 2623-2636.
16. J. West, M. Becker, S. S. Tombrink and A. Manz, *Micro total analysis systems: Latest achievements*. Analytical Chemistry, 2008. **80**(12): p. 4403-4419.
17. C. M. Ferguson, B. G. Coote, N. J. Ashbolt and I. M. Stevenson, *Relationships between indicators, pathogens and water quality in an estuarine system*. Water Research, 1996. **30**(9): p. 2045-2054.
18. K. R. Calci, W. Burkhardt, W. D. Watkins and S.R. Rippey, *Occurrence of male-specific bacteriophage in feral and domestic animal wastes, human feces, and human-associated wastewaters*. Applied and Environmental Microbiology, 1998. **64**(12): p. 5027-5029.
19. A. E. Duran, M. Muniesa, X. Méndez, F. Valero, F. Lucena and F. J. Jofre, *Removal and inactivation of indicator bacteriophages in fresh waters*. Journal of Applied Microbiology, 2002. **92**(2): p. 338-347.
20. A. H. Havelaar, M. van Olphen, and Y. C. Drost, *F-specific RNA bacteriophages are adequate model organisms for enteric viruses in fresh water*. Appl. Environ. Microbiol. , 1993. **59**: p. 2956.
21. W. O. Grabow, C. G. Clay, W. Dhaliwa, M. A. Very and E. E. Müller, *Elimination of viruses, phages, bacteria and Cryptosporidium by a new generation Aquaguard point-of-use water treatment unit*. Zentralblatt Fur Hygiene Und Umweltmedizin, 1999. **202**(5): p. 399-410.

22. G. Caruso, E. Crisafi and M. Mancuso, *Development of an enzyme assay for rapid assessment of Escherichia coli in seawaters*. Journal of Applied Microbiology, 2002. **93**(4): p. 548-556.
23. A. Rompre, P. Servais, J. Baudart, M. R. de-Roubin and P. Laurent, *Detection and enumeration of coliforms in drinking water: current methods and emerging approaches*. Journal of Microbiological Methods, 2002. **49**(1): p. 31-54.
24. M. L. Tortorello, *Indicator organisms for safety and quality - Uses and methods for detection: Minireview*. Journal of Aoac International, 2003. **86**(6): p. 1208-1217.
25. J. Calles-Escandon and M. Cipolla, *Diabetes and endothelial dysfunction: A clinical perspective*. Endocrine Reviews, 2001. **22**(1): p. 36-52.
26. K. C. Tan, W. S. Chow, V. H. Ai and K. S. Lam, *Effects of angiotensin II receptor antagonist on endothelial vasomotor function and urinary albumin excretion in type 2 diabetic patients with microalbuminuria*. Diabetes-Metabolism Research and Reviews, 2002. **18**(1): p. 71-76.
27. Y. Ido, D. Carling and N. Ruderman, *Hyperglycemia-induced apoptosis in human umbilical vein endothelial cells - Inhibition by the AMP-activated protein kinase activation*. Diabetes, 2002. **51**(1): p. 159-167.
28. E. Favaro, I. Miceli, B. Bussolati, M. Schmitt-Ney and P. Cavallo Perin, *Hyperglycemia induces apoptosis of human pancreatic islet endothelial cells - Effects of pravastatin on the Akt survival pathway*. American Journal of Pathology, 2008. **173**(2): p. 442-450.
29. L. Wu, G. P. Li, W. Xu, and M. Bachman, *Droplet formation in microchannels under static conditions*. Applied Physics Letters, 2006. **89**(14).
30. M. Y. He, J. S. Kuo, and D. T. Chiu, *Electro-generation of single femtoliter- and picoliter-volume aqueous droplets in microfluidic systems*. Applied Physics Letters, 2005. **87**(3).
31. S. J. Kim, Y. A. Song, P. L. Skipper and J. Han, *Electrohydrodynamic generation and delivery of monodisperse picoliter droplets using a poly(dimethylsiloxane) microchip*. Analytical Chemistry, 2006. **78**(23): p. 8011-8019.

32. O. Ozen, N. Aubry, D. T. Papageorgiou and P. G. Petropoulos, *Monodisperse drop formation in square microchannels*. Physical Review Letters, 2006. **96**(14).
33. T. B. Jones, *On the relationship of dielectrophoresis and electrowetting*. Langmuir, 2002. **18**(11): p. 4437-4443.
34. J. Zeng and T. Korsmeyer, *Principles of droplet electrohydrodynamics for lab-on-a-chip*. Lab on a Chip, 2004. **4**(4): p. 265-277.
35. R. M. Lorenz, J. S. Edgar, G. D. Jeffries and D. T. Chiu, *Microfluidic and optical systems for the on-demand generation and manipulation of single femtoliter-volume aqueous droplets*. Analytical Chemistry, 2006. **78**(18): p. 6433-6439.
36. E. Quevedo, J. Steinbacher and D.T. McQuade, *Interfacial polymerization within a simplified microfluidic device: Capturing capsules*. Journal of the American Chemical Society, 2005. **127**(30): p. 10498-10499.
37. G. F. Christopher and S. L. Anna, *Microfluidic methods for generating continuous droplet streams*. Journal of Physics D-Applied Physics, 2007. **40**(19): p. R319-R336.
38. C. N. Baroud, F. Gallaire and R. Danga, *Dynamics of microfluidic droplets*. Lab on a Chip, 2010. **10**(16): p. 2032-2045.
39. R. Seemann, M. Brinkmann, T. Pfohl and S. Herminghaus, *Droplet based microfluidics*. Reports on Progress in Physics, 2012. **75**(1).
40. R. Dreyfus, P. Tabeling and H. Willaime, *Ordered and disordered patterns in two-phase flows in microchannels*. Physical Review Letters, 2003. **90**(14).
41. S. L. Anna, N. Bontoux and H.A. Stone, *Formation of dispersions using "flow focusing" in microchannels*. Applied Physics Letters, 2003. **82**(3): p. 364-366.
42. Z. T. Cygan, J. T. Cabral, K. L. Beers and E. J. Amis, *Microfluidic platform for the generation of organic-phase microreactors*. Langmuir, 2005. **21**(8): p. 3629-3634.
43. P. Garstecki, I. Gitlin¹, W. DiLuzio¹, G. M. Whitesides and E. Kumacheva, *Formation of monodisperse bubbles in a microfluidic flow-focusing device*. Applied Physics Letters, 2004. **85**(13): p. 2649-2651.

44. T. P. Lagus and J. F. Edd, *A review of the theory, methods and recent applications of high-throughput single-cell droplet microfluidics*. Journal of Physics D-Applied Physics, 2013. **46**(11).
45. P. Garstecki, *Formation of droplets and bubbles in a microfluidic T-junction - scaling and mechanism of break-up*. Lab on a Chip, 2006. **6**(3): p. 437-446.
46. A. R. Abate, P. Mary, V. van Steijn and D. A. Weitz DA, *Experimental validation of plugging during drop formation in a T-junction*. Lab on a Chip, 2012. **12**(8): p. 1516-1521.
47. T. Nisisako, T. Torii and T. Higuchi, *Droplet formation in a microchannel network*. Lab on a Chip, 2002. **2**(1): p. 24-26.
48. C. Priest, S. Herminghaus and R. Seemann, *Generation of monodisperse gel emulsions in a microfluidic device*. Applied Physics Letters, 2006. **88**(2).
49. T. Thorsen, R. W. Roberts, F. H. Arnold and S. R. Quake, *Dynamic pattern formation in a vesicle-generating microfluidic device*. Physical Review Letters, 2001. **86**(18): p. 4163-4166.
50. Tice, J.D. H. Song, A. D. Lyon and F. R. Ismagilov, *Formation of droplets and mixing in multiphase microfluidics at low values of the Reynolds and the capillary numbers*. Langmuir, 2003. **19**(22): p. 9127-9133.
51. A. S. Utada, A. Fernandez-Nieves, A. H. Stone and D. A. Weitz, *Dripping to jetting transitions in coflowing liquid streams*. Physical Review Letters, 2007. **99**(9).
52. J. Bibette, F. L. Calderon and P. Poulin, *Emulsions: basic principles*. Reports on Progress in Physics, 1999. **62**(6): p. 969-1033.
53. H. Song, J. D. Tice and R. F. Ismagilov, *A microfluidic system for controlling reaction networks in time*. Angewandte Chemie-International Edition, 2003. **42**(7): p. 768-772.
54. E. M. Chan, A. P. Alivisatos and R. A. Mathies, *High-temperature microfluidic synthesis of CdSe nanocrystals in nanoliter droplets*. Journal of the American Chemical Society, 2005. **127**(40): p. 13854-13861.

55. D. L. Chen, C. J. Gerdtz and R. F. Ismagilov, *Using microfluidics to observe the effect of mixing on nucleation of protein crystals*. Journal of the American Chemical Society, 2005. **127**(27): p. 9672-9673.
56. B. Zheng and R. F. Ismagilov, *A microfluidic approach for screening submicroliter volumes against multiple reagents by using preformed arrays of nanoliter plugs in a three-phase liquid/liquid/gas flow*. Angewandte Chemie-International Edition, 2005. **44**(17): p. 2520-2523.
57. L. H. Hung, K. M. Choi, W. Y. Tseng, Y. C. Tan and A. P. Lee, *Alternating droplet generation and controlled dynamic droplet fusion in microfluidic device for CdS nanoparticle synthesis*. Lab on a Chip, 2006. **6**(2): p. 174-178.
58. K. Handique and M. A. Burns, *Mathematical modeling of drop mixing in a slit-type microchannel*. Journal of Micromechanics and Microengineering, 2001. **11**(5): p. 548-554.
59. D. Chatterjee, B. Hetayothin, A. R. Wheeler and D. J. King and R. L. Garrell, *Droplet-based microfluidics with nonaqueous solvents and solutions*. Lab on a Chip, 2006. **6**(2): p. 199-206.
60. A. R. Wheeler, H. Moon, C. J. Kim, J. A. Loo and R. L. Garrell, *Electrowetting-based microfluidics for analysis of peptides and proteins by matrix-assisted laser desorption/ionization mass spectrometry*. Analytical Chemistry, 2004. **76**(16): p. 4833-4838.
61. A. D. Griffiths and D. S. Tawfik, *Miniaturising the laboratory in emulsion droplets*. Trends in Biotechnology, 2006. **24**(9): p. 395-402.
62. J. H. Leamon, D. R. Link, M. Egholm and J. M. Rothberg, *Overview: Methods and applications for droplet compartmentalization of biology*. Nature Methods, 2006. **3**(7): p. 541-543.
- A. Liau, R. Karnik, A. Majumdar and J. H. Cate, *Mixing crowded biological solutions in milliseconds*. Analytical Chemistry, 2005. **77**(23): p. 7618-7625.
64. L. S. Roach, H. Song, and R. F. Ismagilov, *Controlling nonspecific protein adsorption in a plug-based microfluidic system by controlling interfacial chemistry using fluororous-phase surfactants*. Analytical Chemistry, 2005. **77**(3): p. 785-796.

65. N. T. Nguyen, *Interfacial tension measurement with an optofluidic sensor*. Ieee Sensors Journal, 2007. **7**(5-6): p. 692-697.
66. Y. Yamamoto and R. E. Slusher, *Optical Processes in Microcavities*. Physics Today, 1993. **46**(6): p. 66-73.
67. S. K. Tang, R. Derda, Q. Quan, M. Lončar and G. M. Whitesides *Continuously tunable microdroplet-laser in a microfluidic channel*. Optics Express, 2011. **19**(3): p. 2204-2215.
68. S. K. Tang, Z. Li, A. R. Abate, J. J. Agresti, D. A. Weitz, D. Psaltis and G. M. Whitesides, *A multi-color fast-switching microfluidic droplet dye laser*. Lab on a Chip, 2009. **9**(19): p. 2767-2771.
69. M. Tanyeri, R. Perron, and I. M. Kennedy, *Lasing droplets in a microfabricated channel*. Optics Letters, 2007. **32**(17): p. 2529-2531.
70. Teh, S.Y, R. Lin, L. H. Hung and A. P. Lee, *Droplet microfluidics*. Lab on a Chip, 2008. **8**(2): p. 198-220.
71. W. Lee, Y. Sun, H. Li, K. Reddy, M. Sumetsky and X. Fan, *A quasi-droplet optofluidic ring resonator laser using a micro-bubble*. Applied Physics Letters, 2011. **99**(9).
72. W, Lee, Y. Luo, Q. Zhu and X. Fan, *Versatile optofluidic ring resonator lasers based on microdroplets*. Optics Express, 2011. **19**(20): p. 19668-19674.
73. P. Muller, A. Kloss, P. Liebetraut, W. Mönch and H. Zappe, *A fully integrated optofluidic attenuator*. Journal of Micromechanics and Microengineering, 2011. **21**(12).
74. H. W. Ren and S. T. Wu, *Optical switch using a deformable liquid droplet*. Optics Letters, 2010. **35**(22): p. 3826-3828.
75. C. U. Murade, J. M. Oh, D. van den Ende and F. Mugele, *Electrowetting driven optical switch and tunable aperture*. Optics Express, 2011. **19**(16): p. 15525-15531.
76. L. K. Chin, A. Q. Liu, J. B. Zhang, C. S. Lim, Y. C. Soh, *An on-chip liquid tunable grating using multiphase droplet microfluidics*. Applied Physics Letters, 2008. **93**(16).

77. M. Hashimoto, B. Mayers, P. Garstecki, G. M. Whitesides, *Flowing lattices of bubbles as tunable, self-assembled diffraction gratings*. *Small*, 2006. **2**(11): p. 1292-1298.
78. M. Chabert and J. L. Viovy, *Microfluidic high-throughput encapsulation and hydrodynamic self-sorting of single cells*. *Proceedings of the National Academy of Sciences of the United States of America*, 2008. **105**(9): p. 3191-3196.
79. T. P. Lagus and J. F. Edd, *High throughput single-cell and multiple-cell micro-encapsulation*. *J Vis Exp*, 2012. **15**(64): p. 4096.
80. Y. S. Lin, C. H. Yang, K. Lu, K. S. Huang and Y. Z. Zheng, *Synthesis of agar microparticles using temperature-controlled microfluidic devices for Cordyceps militaris cultivation*. *Electrophoresis*, 2011. **32**(22): p. 3157-3163.
81. Y. J. Eun, A. S. Utada, M. F. Copeland, S. Takeuchi and D. B. Weibel, *Encapsulating Bacteria in Agarose Microparticles Using Microfluidics for High-Throughput Cell Analysis and Isolation*. *Acs Chemical Biology*, 2011. **6**(3): p. 260-266.
82. K. C. Ng, K. H. Heredia and D. Kliever, *Note: A method to isolate and detect a large number of single molecules by microdroplet fluorescence spectroscopy*. *Review of Scientific Instruments*, 2012. **83**(3).
83. J. C. Baret, O. J. Miller, V. Taly, M. Ryckelynck, A. El-Harrak, et al., *Fluorescence-activated droplet sorting (FADS): efficient microfluidic cell sorting based on enzymatic activity*. *Lab on a Chip*, 2009. **9**(13): p. 1850-1858.
84. A. Y. Lau, L. P. Lee, and J. W. Chan, *An integrated optofluidic platform for Raman-activated cell sorting*. *Lab on a Chip*, 2008. **8**(7): p. 1116-1120.
85. Y. Yang, A. Q. Liu, L. Lei, L. K. Chin, C. D. Ohl, Q. J. Wang, H. S. Yoon, *A tunable 3D optofluidic waveguide dye laser via two centrifugal Dean flow streams*. *Lab Chip*. 2011. **11**(18) : p. 3182-3187
86. Ernst, A., W. Streule, N. Schmitt, R. Zengerle and P. Koltaym, *A capacitive sensor for non-contact nanoliter droplet detection*. *Sensors and Actuators a-Physical*, 2009. **153**(1): p. 57-63.

87. C. Elbuken, T. Glawdel, D. Chan, C. L. Ren, *Detection of microdroplet size and speed using capacitive sensors*. Sensors and Actuators a-Physical, 2011. **171**(2): p. 55-62.
88. Y. Q. Lin, Y. Lin, S. Schiavo, J. Orjala, P. Vouros and R. Kautz, *Microscale LC-MS-NMR Platform Applied to the Identification of Active Cyanobacterial Metabolites*. Analytical Chemistry, 2008. **80**(21): p. 8045-8054.
89. J. Madsen and S. P. Armes, *(Meth)acrylic stimulus-responsive block copolymer hydrogels*. Soft Matter, 2012. **8**(3): p. 592-605.
90. S. Salehpour and M. A. Dube, *Application Properties of Stimuli-Responsive Polyglycerol Hydrogels*. Journal of Macromolecular Science Part a-Pure and Applied Chemistry, 2012. **49**(2): p. 103-110.
91. A. W. Bigelow, D. J. Brenner, G. Garty and G. Randers-Pehrson, *Single-particle/single-cell ion microbeams as probes of biological mechanisms*. Ieee Transactions on Plasma Science, 2008. **36**(4): p. 1424-1431.
92. M. Grad, A. W. Bigelow, G. Garty, D. Attinger and D. J. Brenner, *Optofluidic cell manipulation for a biological microbeam*. Review of Scientific Instruments, 2013. **84**(1).
93. T. Chen, L. Z. Shi, Q. Zhu, C. Chandsawangbhuwana and M. W. Berns, *Optical tweezers and non-ratiometric fluorescent-dye-based studies of respiration in sperm mitochondria*. Journal of Optics, 2011. **13**(4).
94. S. C. Santucci, D. Cojoc, H. Amenitsch, B. Marmioli, B. Sartori, et al., *Optical Tweezers for Synchrotron Radiation Probing of Trapped Biological and Soft Matter Objects in Aqueous Environments*. Analytical Chemistry, 2011. **83**(12): p. 4863-4870.
95. X. Heng X, D. Erickson, L. R. Baugh, Z. Yaqoo, P. W. Sternberg, et al., *Optofluidic microscopy - a method for implementing a high resolution optical microscope on a chip*. Lab on a Chip, 2006. **6**(10): p. 1274-1276.
96. N. Van Gerven, V. Deros and J. P. Hernalsteens, *A microfluidic shadow imaging system for the study of the nematode Caenorhabditis elegans in space*. Sensors and Actuators B-Chemical, 2005. **107**(2): p. 904-914.

97. S. H. Cho, J. M. Godin, C. H. Chen, W. Qiao, H. Lee, Y. H. Lo, *Review Article: Recent advancements in optofluidic flow cytometer*. *Biomicrofluidics*, 2010. **4**(4).
98. M. Keeney, I. Chin-Yee, K. Weir, J. Popma, R. Nayar and D. R. Sutherland, *Single platform flow cytometric absolute CD34+ cell counts based on the ISHAGE guidelines*. *Cytometry*, 1998. **34**(2): p. 61-70.
99. S. H. Cho, C. H. Chen, F. S. Tsai, J. M. Godin and Y. H. Lo, *Human mammalian cell sorting using a highly integrated micro-fabricated fluorescence-activated cell sorter (mu FACS)*. *Lab on a Chip*, 2010. **10**(12): p. 1567-1573.
100. J. Crawford, D. C. Dale, and G. H. Lyman, *Chemotherapy-induced neutropenia - Risks, consequences, and new directions for its management*. *Cancer*, 2004. **100**(2): p. 228-237.
101. T. F. Wu, Z. Mei, and Y.H. Lo, *Optofluidic device for label-free cell classification from whole blood*. *Lab on a Chip*, 2012. **12**(19): p. 3791-3797.
102. P. Fei, Z. Chen, Y. Men, A. Li, Y. Shen, et al., *A compact optofluidic cytometer with integrated liquid-core/PDMS-cladding waveguides*. *Lab on a Chip*, 2012. **12**(19): p. 3700-3706.
103. M. Kukkula, L. Maunula, E. Silvennoinen, C. H. von Bonsdorff, *Outbreak of viral gastroenteritis due to drinking water contaminated by Norwalk-like viruses*. *Journal of Infectious Diseases*, 1999. **180**(6): p. 1771-1776.
104. J. F. Schijven, P. F. Teunis, S. A. Rutjes, M. Bouwknecht and A. M. de Roda Husman, *QMRAspot: A tool for Quantitative Microbial Risk Assessment from surface water to potable water*. *Water Research*, 2011. **45**(17): p. 5564-5576.
105. L. Ogorzaly and C. Gantzer, *Development of real-time RT-PCR methods for specific detection of F-specific RNA bacteriophage genogroups: Application to urban raw wastewater*. *Journal of Virological Methods*, 2006. **138**(1-2): p. 131-139.
106. R. Cornax R, M. A. Moriño MA, I. G. Paez, M. A. Muñoz, J. J. Borrego, *Applicaition of direct plaque-assay for detection and enumeration of bacteriophages of baterioides-fragilis from contaminated-water samples*. *Applied and Environmental Microbiology*, 1990. **56**(10): p. 3170-3173.

107. M. Gabig-Ciminska, M. Los, A. Holmgren, J. Albers, A. Czyz A, et al., *Detection of bacteriophage infection and prophage induction in bacterial cultures by means of electric DNA chips*. Analytical Biochemistry, 2004. **324**(1): p. 84-91.
108. F. N. Dultsev, R. E. Speight, M. T. Fiorini, J. M. Blackburn, C. Abell, et al., Klenerman D *Direct and quantitative detection of bacteriophage by "Hearing" surface detachment using a quartz crystal microbalance*. Analytical Chemistry, 2001. **73**(16): p. 3935-3939.
109. M. He, J. S. Edgar, G. D. Jeffries, R. M. Lorenz, J. P. Shelby, et al, *Selective encapsulation of single cells and subcellular organelles into picoliter- and femtoliter-volume droplets*. Analytical Chemistry, 2005. **77**(6): p. 1539-1544.
110. E. Brouzes, M. Medkovaa, N. Savenellia, D. Marrana, M. Twardowski, *Droplet microfluidic technology for single-cell high-throughput screening*. Proceedings of the National Academy of Sciences of the United States of America, 2009. **106**(34): p. 14195-14200.
111. H. N. Joensson and H. A. Svahn, *Droplet Microfluidics-A Tool for Single-Cell Analysis*. Angewandte Chemie-International Edition, 2012. **51**(49): p. 12176-12192.
112. T. D. Rane, H. C. Zec, C. Puleo, A. P. Lee, T. H. Wang, *Droplet microfluidics for amplification-free genetic detection of single cells*. Lab on a Chip, 2012. **12**(18): p. 3341-3347.
113. W. Z. Song, A. Q. Liu, S. Swaminathan, C. S. Lim, P. H. Yap and T. C. Ayi, *Determination of single living cell's dry/water mass using optofluidic chip*. Applied Physics Letters, 2007. **91**(22).
114. N. Riefler, R. Schuh and T. Wriedt, *Investigation of a measurement technique to estimate concentration and size of inclusions in droplets*. Measurement Science & Technology, 2007. **18**(7): p. 2209-2218.
115. T. Wriedt and R. Schuh, *The inclusion-concentration measurement of suspension droplets based on Monte Carlo ray tracing*. Measurement Science & Technology, 2002. **13**(3): p. 276-279.

116. J. Fattaccioli, J. Baudry, J. Émerard, E. Bertrand, C. Goubault, et al., *Size and fluorescence measurements of individual droplets by flow cytometry*. Soft Matter, 2009. **5**(11): p. 2232-2238.
117. A. Calver, J. Collier and P. Vallance, *Inhibition and stimulation of nitric-oxide synthesis in the human forearm arterial bed of patients with insulin-depedent diabetes* Journal of Clinical Investigation, 1992. **90**(6): p. 2548-2554.
118. J. M. Hill, G. Zalos, J. P. Halcox, W. H. Schenke, M. A. Waclawiw, et al., *Circulating endothelial progenitor cells, vascular function, and cardiovascular risk*. New England Journal of Medicine, 2003. **348**(7): p. 593-600.
119. M. L. Sheu, C. K. Chiang CK, K. S. Tsai, F. M. Ho and Weng TI, *Inhibition of NADPH oxidase-related oxidative stress-triggered signaling by honokiol suppresses high glucose-induced human endothelial cell apoptosis*. Free Radical Biology and Medicine, 2008. **44**(12): p. 2043-2050.
120. Y. Takamura, T. Tomomatsu, E. Kubo, S. Tsuzuki, Y. Akagi, *Role of the polyol pathway in high glucose-induced apoptosis of retinal Pericytes and proliferation of endothelial cells*. Investigative Ophthalmology & Visual Science, 2008. **49**(7): p. 3216-3223.
121. I. A. van den Oever , H. G. Raterman, M. T. Nurmohamed, S. Simsek, *Endothelial Dysfunction, Inflammation, and Apoptosis in Diabetes Mellitus*. Mediators of Inflammation, 2010.
122. H. Ishii, D. Koya, and G. L. King, *Protein kinase C activation and its role in the development of vascular complications in diabetes mellitus*. Journal of Molecular Medicine-Jmm, 1998. **76**(1): p. 21-31.
123. L. Quagliaro, L. Piconi, R. Assaloni, L. Martinelli, E. Mot and A. Ceriello, *Intermittent high glucose enhances apoptosis related to oxidative stress in human umbilical vein endothelial cell - The role of protein kinase C and NAD(P)H-oxidase activation*. Diabetes, 2003. **52**(11): p. 2795-2804.
124. S. Srinivasan, M. E. Hatley, D. T. Bolick, L. A. Palmer, D. Edelstein, et al., *Hyperglycaemia-induced superoxide production decreases eNOS expression via AP-1 activation in aortic endothelial cells*. Diabetologia, 2004. **47**(10): p. 1727-1734.

125. J. S. Armstrong and M. Whiteman, *Measurement of reactive oxygen species in cells and mitochondria*, in *Mitochondria, 2nd Edition*, L.A. Pon and E.A. Schon, Editors. 2007. p. 355-377.
126. T. Finkel and N. J. Holbrook, *Oxidants, oxidative stress and the biology of ageing*. Nature, 2000. **408**(6809): p. 239-247.
127. L. G. Kevin, E. Novalija and D. F. Stowe, *Reactive oxygen species as mediators of cardiac injury and protection: The relevance to anesthesia practice*. Anesthesia and Analgesia, 2005. **101**(5): p. 1275-1287.
128. M. Giorgio, M. Trinei, E. Migliaccio and P. G. Pelicci, *Hydrogen peroxide: a metabolic by-product or a common mediator of ageing signals?* Nature Reviews Molecular Cell Biology, 2007. **8**(9): p. 722A-728.
129. D. R. Green and G. Kroemer, *The pathophysiology of mitochondrial cell death*. Science, 2004. **305**(5684): p. 626-629.
130. T. Yu, S. S. Sheu, J. L. Robotham and Y. Yoon, *Mitochondrial fission mediates high glucose-induced cell death through elevated production of reactive oxygen species*. Cardiovascular Research, 2008. **79**(2): p. 341-351.
131. J. B. Brocard, G. L. Rintoul, and I. J. Reynolds, *New perspectives on mitochondrial morphology in cell function*. Biology of the Cell, 2003. **95**(5): p. 239-242.
132. L. L. Ji, *Antioxidants and oxidative stress in exercise*. Proceedings of the Society for Experimental Biology and Medicine, 1999. **222**(3): p. 283-292.
133. A. W. Cowley, T. Mori, D. Mattson and A. P. Zou, *Role of renal NO production in the regulation of medullary blood flow*. American Journal of Physiology-Regulatory Integrative and Comparative Physiology, 2003. **284**(6): p. R1355-R1369.
134. T. Inoguchi, T. Sonta, H. Tsubouchi, T. Etoh, M. Kakimoto, et al., *High glucose level and free fatty acid stimulate reactive oxygen species production through protein kinase C-dependent activation of NAD(P)H oxidase in cultured vascular cells*. Diabetes, 2000. **49**(11): p. 1939-1945.
135. T. Matsuda, K. Omori, T. Vuong, M. Pascual and L. Valiente, *Inhibition of p38 pathway suppresses human islet production of pro-inflammatory cytokines and*

- improves islet graft function*. American Journal of Transplantation, 2005. **5**(3): p. 484-493.
136. O. I. Aruoma, *Free radicals, oxidative stress, and antioxidants in human health and disease*. Journal of the American Oil Chemists Society, 1998. **75**(2): p. 199-212.
 137. T. Sano, F. Umeda, T. Hashimoto, H. Nawata and H. Utsumi, *Oxidative stress measurement by in vivo electron spin resonance spectroscopy in rats with streptozotocin-induced diabetes*. Diabetologia, 1998. **41**(11): p. 1355-1360.
 138. Z. Zhang, N. I. Dmitrieva, J. H. Park, R. L. Levine, M. B. Burg, *High urea and NaCl carbonylate proteins in renal cells in culture and in vivo, and high urea causes 8-oxoguanine lesions in their DNA*. Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(25): p. 9491-9496.
 139. H. J. Hsieh, C. C. Cheng, S. T. Wu, J. J. Chiu JJ, B. S. Wung, et al., *Increase of reactive oxygen species (ROS) in endothelial cells by shear flow and involvement of ROS in shear-induced c-fos expression*. Journal of Cellular Physiology, 1998. **175**(2): p. 156-162.
 140. H. H. Lipowsky, S. Usami, and S. Chien, *In vivo measurements of hematocrit and apparent viscosity in the microvasculature of cat mesentery*. Microvasc Res 1980. **19**: p. 297-319.
 141. H. H. Lipowsky, K. S, and B.W. Zweifach, *The distribution of blood rheological parameters in the microcirculation of cat mesentery*. Circ Res 1978. **43**: p. 738-749.
 142. N. Resnick, H. Yahav, A. Shay-Salit, M. Shushy, S. Schubert, et al., *Fluid shear stress and the vascular endothelium: for better and for worse*. Progress in Biophysics & Molecular Biology, 2003. **81**(3): p. 177-199.
 143. O. Tricot, Z. Mallat, C. Heymes, J. Belmin, G. Lesèche, et al., *Relation between endothelial cell apoptosis and blood flow direction in human atherosclerotic plaques*. Circulation, 2000. **101**(21): p. 2450-2453.
 144. X. Bai, X. Wang, and Q. B. Xu, *Endothelial damage and stem cell repair in atherosclerosis*. Vascular Pharmacology, 2010. **52**(5-6): p. 224-229.

145. D. van Noort, S. M. Ong, C. Zhang, S. Zhang, T. Arooz, et al., *Stem Cells in Microfluidics*. Biotechnology Progress, 2009. **25**(1): p. 52-60.
146. C. F. Dewey, S. R. Bussolari, M. A. Gimbrone, P. F. Davies. *The dynamic response of vascular endothelial cell to fluid shear-stress*. Journal of Biomechanical Engineering-Transactions of the Asme, 1981. **103**(3): p. 177-185.
147. G. Helmlinger, R. V. Geiger, S. Schreck, R. M. Nerem, *Effects of pulsatile flow on cultured vascular endothelial cell morphology*. Journal of Biomechanical Engineering-Transactions of the Asme, 1991. **113**(2): p. 123-131.
148. J. Rossi, P. Jonak, L. Rouleau, L. Danielczak, J. C. Tardif, et al., *Differential Response of Endothelial Cells to Simvastatin When Conditioned with Steady, Non-Reversing Pulsatile or Oscillating Shear Stress*. Annals of Biomedical Engineering, 2011. **39**(1): p. 402-413.
149. M. Nakamura, W. Shigeo, M. Taisei, K. Akira, K. Takeshi, *A computational fluid mechanical study on the effects of opening and closing of the mitral orifice on a transmitral flow velocity profile and an early diastolic intraventricular flow*. Jsme International Journal Series C-Mechanical Systems Machine Elements and Manufacturing, 2002. **45**(4): p. 913-922.
150. K. A. Barbee, P. F. Davies, and R. Lal, *Shear stress-induces reorganization of the surface-topography of living endothelial cells imaged by atomic-force microscopy*. Circulation Research, 1994. **74**(1): p. 163-171.
151. S. Elmore, *Apoptosis: A review of programmed cell death*. Toxicologic Pathology, 2007. **35**(4): p. 495-516.
152. T. Enoch and C. Norbury, *Cellular-responses to DNA-damage cell cycle checkpoints apoptosis and the roles of P53 and ATM*. Trends in Biochemical Sciences, 1995. **20**(10): p. 426-430.
153. E. Plantin-Carrenard, A. Bringuier, C. Derappe, J. Pichon and R. Guillot, et al., *A fluorescence microplate assay using yopro-1 to measure apoptosis: Application to HL60 cells subjected to oxidative stress*. Cell Biology and Toxicology, 2003. **19**(2): p. 121-133.
154. R. Sgonc and J. Gruber, *Apoptosis detection: An overview*. Experimental Gerontology, 1998. **33**(6): p. 525-533.

155. G. Hacker, *The morphology of apoptosis*. Cell and Tissue Research, 2000. **301**(1): p. 5-17.
156. J. F. Kerr, A. H. Wyllie, and A.R. Currie, *Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics*. Br J Cancer 1972. **26**: p. 239-257.
157. R. Howden, P. R. Hanlon, J. G. Petranka, S. Kleeberger, J. Bucher, et al., *Ephedrine plus caffeine causes age-dependent cardiovascular responses in Fischer 344 rats*. American Journal of Physiology-Heart and Circulatory Physiology, 2005. **288**(5): p. H2219-H2224.
158. M. Kressel and P. Groscurth, *Distinction of apoptotic and necrotic cell-death by in-situ labelling of fragmented DNA*. Cell and Tissue Research, 1994. **278**(3): p. 549-556.
159. A. R. Clapp, I. L. Medintz, J. M. Mauro, B. R. Fisher, M. G. Bawendi, et al., *Fluorescence resonance energy transfer between quantum dot donors and dye-labeled protein acceptors*. Journal of the American Chemical Society, 2004. **126**(1): p. 301-310.
160. R. M. Clegg, *Fluorescence resonance energy transfer and nucleic-acids*. Methods in Enzymology, 1992. **211**: p. 353-388.
161. G. W. Gordon, G. Berry, X. H. Liang, B. Levine and B. Herman, *Quantitative fluorescence resonance energy transfer measurements using fluorescence microscopy*. Biophysical Journal, 1998. **74**(5): p. 2702-2713.
162. F. J. Meng and F. Sachs, *Visualizing dynamic cytoplasmic forces with a compliance-matched FRET sensor*. Journal of Cell Science, 2011. **124**(2): p. 261-269.
163. C. Grashoff, B. D. Hoffman, M. D. Brenner, R. Zhou, M. Parsons, et al., *Measuring mechanical tension across vinculin reveals regulation of focal adhesion dynamics*. Nature, 2010. **466**(7303): p. 263-U143.
164. S. Iwai, and T. Q. P. Uyeda, *Visualizing myosin-actin interaction with a genetically-encoded fluorescent strain sensor*. Proceedings of the National Academy of Sciences of the United States of America, 2008. **105**(44): p. 16882-16887.

165. F. Meng, T. M. Suchyna and F. Sachs, *A fluorescence energy transfer-based mechanical stress sensor for specific proteins in situ*. Febs Journal, 2008. **275**(12): p. 3072-3087.
166. F. J. Meng and F. Sachs, *Orientation-based FRET sensor for real-time imaging of cellular forces*. Journal of Cell Science, 2012. **125**(3): p. 743-750.
167. B. Angres, H. Steuer, P. Weber, M. Wagner and H. Schneckeburger, *A Membrane-Bound FRET-Based Caspase Sensor for Detection of Apoptosis Using Fluorescence Lifetime and Total Internal Reflection Microscopy*. Cytometry Part A, 2009. **75A**(5): p. 420-427.
168. R. A. Figueroa, V. Ramberg, T. Gatsinzi, M. Samuelsson, M. Zhang, et al., *Anchored FRET sensors detect local caspase activation prior to neuronal degeneration*. Molecular Neurodegeneration, 2011. **6**.
169. E. M. Purcell, *Life at low reynolds-number*. American Journal of Physics, 1977. **45**(1): p. 3-11.
170. M. Joanicot and A. Ajdari, *Applied physics - Droplet control for microfluidics*. Science, 2005. **309**(5736): p. 887-888.
171. J. A. B. Faria, *A polarimetric optical microrotation sensor employing a birefringent uniaxial crystal*. Microwave and Optical Technology Letters, 1991. **4**(9): p. 350-354.
172. J. K. Chang, H. Bang, S.-J. Park, S. Chung, C. Chung, et al., *Fabrication of the PDMS microchip for serially diluting sample with buffer*, icrosystem Technologies, 2008, **9**(3): p. 555-558.
173. R. C. Loehr and D. Schwegle, *Filtration method for bactriophage detection*. Applied Microbiology, 1965. **13**(6): p. 1005-&.
174. R. Schuh and T. Wriedt, *Computer programs for light scattering by particles with inclusions*. Journal of Quantitative Spectroscopy & Radiative Transfer, 2001. **70**(4-6): p. 715-723.
175. D. S. Burz, D. Beckett, N. Benson and G. K. Ackers, *Self-assembly of bacteriophage-lambda CI-repressor, effects of single-site mutations on the monomer-dimer equilibrim*. Biochemistry, 1994. **33**(28): p. 8399-8405.

176. R. E. Enns, A. M. Garland and D. W. Smith, *M13-oric cloning vehicles-use for amplification of high copy lethal (HCL) genetic elements*. Plasmid, 1986. **15**(2): p. 147-155.
177. K. Skarstad, H. B. Steen and E. Boye, *Cell-cycle parameters of slowly growing Escherichia Coli B/R studied by flow cytometry*. Journal of Bacteriology, 1983. **154**(2): p. 656-662.
178. R. C. Gonzalez and R. E. Woods, *Digital Image Processing*. Wiley-Interscience Publication, 2007.
179. H. Hotelling, *Analysis of a complex of statistical variables into principal components*. Journal of Educational Psychology, 1933. **24**: p. 417-441.
180. S. Wold, K. Esbensen and P. Geladi, *Principle Component Analysis*. Chemometrics and Intelligent Laboratory Systems, 1987. **2**(1-3): p. 37-52.
181. A. L. Price, N. J. Patterson R. M. Plenge, M. E. Weinblatt, N. A. Shadick, et al., *Principal components analysis corrects for stratification in genome-wide association studies*. Nature Genetics, 2006. **38**(8): p. 904-909.
182. R. C. Gonzalez and R. E. Woods, *Digital image processing using MATLAB*. Wiley-Interscience Publication, 2007.
183. L. Cai, W. Li, G. Wang, L. Guo, Y. Jiang, et al., *Hyperglycemia-induced apoptosis in mouse myocardium - Mitochondrial cytochrome c-mediated caspase-3 activation pathway*. Diabetes, 2002. **51**(6): p. 1938-1948.
184. L. K. Chin, A. Q. Liu, Y. C. Soh, C. S. Lim and C. L. Lin. *A reconfigurable optofluidic Michelson interferometer using tunable droplet grating*. Lab on a Chip, 2010. **10**(8): p. 1072-1078.
185. D. N. Ku , *Blood flow in arteries*. Annual Review of Fluid Mechanics, 1997. **29**: p. 399-434.
186. X. Y. He , D. N. Ku and J. E. Moore, *Simple calculation of the velocity profiles for pulsatile flow in a blood-vessel using methmatica*. Annals of Biomedical Engineering, 1993. **21**(1): p. 45-49.
187. D. J. Duncker, R. J. Bache and D. Merkus, *Regulation of coronary resistance vessel tone in response to exercise*. Journal of Molecular and Cellular Cardiology, 2012. **52**(4): p. 802-813.

188. H. Lu, L. Y. Koo, W. M. Wang, D. A. Lauffenburger and L. G. Griffith, *Microfluidic shear devices for quantitative analysis of cell adhesion*. Analytical Chemistry, 2004. **76**(18): p. 5257-5264.
189. A. Castro, J. M. del Corral, M. Gordaliza, C. Grande, A. Gómez-Zurita, *Synthesis and cytotoxicity of podophyllotoxin analogues modified in the A ring*. European Journal of Medicinal Chemistry, 2003. **38**(1): p. 65-74.
190. W. Dai, Y. Zheng, K. Q. Luo, H. Wu, *A prototypic microfluidic platform generating stepwise concentration gradients for real-time study of cell apoptosis*. Biomicrofluidics, 2010. **4**(2).
191. J. Q. Yu, Y. Yang, A. Q. Liu, L. K. Chin and X. M. Zhang, *Microfluidic droplet grating for reconfigurable optical diffraction*. Optics Letters, 2010. **35**(11): p. 1890-1892.
192. D. Kulms and T. Schwarz, *Molecular mechanisms of UV-induced apoptosis*. Photodermatology Photoimmunology & Photomedicine, 2000. **16**(5): p. 195-201.
193. S. S. Bacus, A. V. Gudkov, M. Lowe, L. Lyass, Y. Yung, et al., *Taxol-induced apoptosis depends on MAP kinase pathways (ERK and p38) and is independent of p53*. Oncogene, 2001. **20**(2): p. 147-155.
194. P. F. Davies, *Flwo-mediated Endothelial mechanotransduction*. Physiological Reviews, 1995. **75**(3): p. 519-560.
195. P. F. Davies and S. C. Tripathi, *Mechanical-stress machnisms and the cell-an endothelial paradigm*. Circulation Research, 1993. **72**(2): p. 239-245.
196. R. K. Jain, N. Safabakhsh, A. Sckell, Y. Chen, P. Jiang, et al., *Endothelial cell death, angiogenesis, and microvascular function after castration in an androgen-dependent tumor: Role of vascular endothelial growth factor*. Proceedings of the National Academy of Sciences of the United States of America, 1998. **95**(18): p. 10820-10825.
197. H. L. Tian, T. Yu, N. N. Xu, C. Feng, L. Y. Zhou, et al., *A novel compound modified from tanshinone inhibits tumor growth in vivo via activation of the intrinsic apoptotic pathway*. Cancer Letters, 2010. **297**(1): p. 18-30.