



Australian
National
University

**ADVANCED VOLUMETRIC
MICROSCOPY TECHNIQUES FOR
DYNAMIC STUDIES OF ANUCLEATE
CELLS**

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Declaration

This thesis is an original work conducted by me during the period of September 2014 to September 2018 at the Research School of Engineering, Australian National University, Canberra, Australia.

This PhD work has been primarily supervised by Dr Woei Ming Lee and partial support supervision by Assoc. Prof. Nisbet David Russell, Assoc. Prof. Maier, Alexander and Assoc. Prof. Elizabeth Gardiner throughout.

Parts of the works in the thesis (Chapter 6, 7) were conducted as collaboration projects with a PhD student, Ms Mrinalini Pratap, in Research School of Biology and Dr Samantha Montague in John Curtin School of Medical Research. Part of software programming was jointly developed with Ms Yujie Zheng, a Honours student in Research School of Engineering. Fabrication of microfluidic devices were done with the help of Mr Tao Xu, a technician in ANU Applied Optics Lab. Each of their contribution(s) have been mentioned in the relevant chapters accordingly.

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Abstract

Optical imaging is a cornerstone in biological and biomedical sciences. It is imperative to not only obtain high-resolution images but also provide real-time quantification of biological measurement using automated non-invasive imaging solutions. In recent years, the advent of volumetric microscopes, especially laser scanning microscopy (LSM) and quantitative phase microscopy (QPM), has opened up new powerful imaging tools for biological studies.

One of the primary biological activities that require volumetric imaging is blood dynamics. Blood is an essential component in the circulatory system, which transports nutrients, oxygen, and waste for the entire body. Anucleate blood cells, red blood cells (RBCs) and platelets, are majority contributors in homeostasis, the state of steady internal conditions, and hemostasis, the process of bleeding control. Any morphological anomalies of RBCs and platelets could result in mortality. For examples, poor deformability of RBCs influenced by blood-borne diseases (e.g. malaria, sickle) can result in poor delivery of nutrients and oxygen to major visceral organs. And excessive or insufficient blood coagulation due to kinds of platelet disorders can lead to thrombosis or abnormal bleeding.

This thesis presents the development of volumetric imaging systems and tailored microfluidics platform for the study of diseased anucleate blood cells. Using a custom-built automated QPM system, quantitative deformability measurement of parasites infected RBCs was conducted using an adhesion assay. Furthermore, real-time QPM imaging of platelets aggregation was achieved for studying thrombus dynamic. Then, a hybrid image system, Holographic inverted Single Plane Illumination Microscopy (H^+iSPIM), was developed to retrieve both structural and molecular information, providing multidimensional images at the same time.

The first chapter introduces the aims and the structure of the thesis. The second, third and fourth chapters presents background of the techniques used in the thesis; volumetric microscopy techniques, biomechanics of anucleate blood cells, and microfluidic respectively. Chapter five elaborates the hardware and software development for an automated DHM system. This chapter is meant to extend the usage of DHM by automated image processing and data analysis. The

next following two chapters (six and seven) describe the deformability studies of infected RBC and dynamics of platelet-platelet aggregate. Chapter eight proposes a hybrid imaging system, H⁺iSPIM, for multidimensional studies of cells in microfluidic systems. Conclusions, future works, and limitations of the developed system are discussed in the final chapter.

In summary, this thesis presents the pursuit of versatile imaging systems for non-invasive imaging quantification of dynamic cellular studies using microfluidic devices for blood-related diseases.

Publications

Journals:

1. **Xuefei He**, Samantha J. Montague, Xu Tao, Elizabeth E. Gardiner, and Woei Ming Lee†. "Quantifying Embolism: Label-Free Volumetric Mapping of Thrombus Structure and Kinesis in a Microfluidic System with Optical Holography." *Advanced Biosystems*: 1800089.
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3. **Xuefei He**, Chuong Vinh Nguyen, Mrinalini Pratap, Yujie Zheng, Yi Wang, David R. Nisbet, Richard J. Williams, Melanie Rug, Alexander G. Maier, and Woei Ming Lee†. "Automated Fourier space region-recognition filtering for off-axis digital holographic microscopy" *Biomedical Optics Express*. 2016 Aug 1;7(8):3111-23.

Conference Proceedings/Talks:

1. Christine S.M. Lee, Samantha J. Montague, Sarah Hicks, **Xuefei He**, Philip Choi, Robert K. Andrews, Woei Ming Lee, Elizabeth E. Gardiner† "Plasma inhibitors of platelet sheddase activity – Rocks to the scissors on the paper", *Blood 2018 Meeting Brisbane* (2018)
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5. **Xuefei He**, Katharina Gaus, and Woei Ming Lee†. Label-free dynamic volumetric imaging of deforming giant unilamellar vesicles under micro-flows. InBio-Optics: Design and Application 2017 Apr 2 (pp. BoS2A-2). Optical Society of America.
6. **Xuefei He**, Yujie Zheng, and Woei Ming Lee†. DHMI: dynamic holographic microscopy interface. InSPIE BioPhotonics Australasia 2016 Dec 9 (Vol. 10013, p. 1001332). International Society for Optics and Photonics.
7. **Xuefei He**, Chuong Vinh Nguyen, Mrinalini Pratap, Yujie Zheng, Yi Wang, David R. Nisbet, Melanie Rug, Alexander G. Maier, and Woei Ming Lee†. Adaptive spatial filtering for off-axis digital holographic microscopy based on region recognition approach with iterative thresholding. InSPIE BioPhotonics Australasia 2016 Dec 9 (Vol. 10013, p. 1001329). International Society for Optics and Photonics.
8. **Xuefei He**, Alexander G. Maier, and Woei Ming Lee†. Mapping the progression of malaria infected erythrocytes with holographic microscopy. InConference on Lasers and Electro-Optics/Pacific Rim 2015 Aug 24 (p. 27P_73). Optical Society of America.

Letter to the editor:

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Nomenclature

Two dimensional	2D
Two and a half dimensional	2.5D
Three dimensional	3D
Four dimensional	4D
Avalanche photodiode	APD
Atomic force microscopy	AFM
Acousto-optic modulators	AOM
3-Aminopropyltriethoxysilane	APES
Charged Coupled Device	CCD
Concanavalin A (carbohydrate-binding protein)	Con-A
confocal laser scanning microscopy (CLSM)	CLSM)
Digital holographic microscopy	DHM
Digital scanned laser light-sheet fluorescence microscopy	DSLm
Dual/double-side inverted selective plane illumination microscopy	diSPIM
direct stochastic optical reconstruction microscopy	dSTORM
depth of field	DOF
Fast Fourier transformation	FFT
Fused Deposition Modeling	FDM
Fused Filament Fabrication	FFF
Field of view	FOV
Full width at half-maximum	FWHM
Global threshold level	GTL
Graphical user interface	GUI
Galvanometer-mounted tilting mirror (GM)	GM
Holographic inverted selective plane illumination microscopy	H⁺iSPIM
Hemoglobin S	HbS
Human immunodeficiency virus	HIV
Inverted selective plane illumination microscopy	iSPIM
Infected red blood cells	iRBCs
Stereolithography	SLA
Scientific Complementary Metal Oxide Semiconductor	sCMOS
Scanning electron microscopy	SEM

Structured illumination microscopy	SIM
Laser scanning microscopy	LSM
Magnetic twisting cytometry	MTC
Multiview selective-plane illumination microscope	MuVi-SPIM
Micropipette aspiration	MA
Multidirectional selective plane illumination microscopy	mSPIM
Parasitophorous vacuole	PV
Parasitophorous vacuolar membrane	PVM
Particle tracking microrheology	PTM
Paroxysmal Nocturnal Hemoglobinuria	PNH
Phosphate-buffered saline	PBS
D-Phenylalanyl-prolyl-arginine Chloromethy Ketone	PPACK
Platelet rich plasma	PRP
Physical Containment Laboratory	PC2
Poly-dimethylsiloxane	PDMS
Quantitative phase microscopy	QPM
Red Blood Cells	RBCs
Reynolds number	Re
Region of interest	ROI
Total internal reflection fluorescence microscope	TIRF
United States Air Force	USAF
Ultraviolet	UV
Von Willebrand disease	(VWD)
Wide-field fluorescence microscopy	WFM

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Chapter 1 Introduction

1.1 Motivation

In the field of biological sciences, the observation and investigation of living specimens at the microscopic size-scale are especially invaluable. Hence, optical microscopy plays an unrivalled role in exploring the insight of biological processes by observing living cells, organs or embryos during development, that would otherwise be impossible. Living biological systems are highly complex. It comprises a host of cascading dynamic interactions related to cellular mechanics well as biochemical signals. Due to these tiny spatial dynamics between cells, real-time three-dimensional quantification plays a significant part in revealing the biomechanical/biochemical properties of cells. Consequently, copious amounts of efforts have been devoted to developing volumetric imaging techniques to satisfy various cellular biology studies¹⁻⁴. Three-dimensional imaging using laser scanning techniques, such as confocal laser scanning microscopy⁵⁻⁶, multiphoton microscopy⁷⁻⁸, light sheet microscopy⁹, and super-resolution microscopes¹⁰⁻¹¹ are the representatives of the frontiers of volumetric imaging techniques. High resolution, depth selectivity and single protein specificity offered by these techniques have accelerated progress in pathological research of diseases at the cellular level¹²⁻¹³, which complements other biochemical research¹⁴⁻¹⁵, single molecule¹⁶ for last decades. However, one of drawbacks in fluorescence microscopy is requirement to selectively stain samples. Sample preparation often involves exogenous fluorophore which usually need to attach to another molecule before it can be attached to a cell. This alteration could potentially affects cells' natural state¹⁷⁻¹⁸. On the other hand, there are more detrimental effects such as photobleaching and phototoxicity. Direct laser irradiation for efficient fluorescence excitation have shown to impair cell functions and reduce its viability for long-term investigation^{17, 48}. Emerging techniques to overcome phototoxicity in fluorescent imaging include light sheet microscopy and confocal spinning disc microscopy. Without fluorescent-labelling, it is also possible to capture sub-cellular structures using phase-based light microscopy. Quantitative phase microscopy (QPM)¹⁹⁻²² has emerged into a powerful tool for label-free live cells study. It offers a two-dimensional height map of the sample by retrieving the

phase delay of the light passing through the sample. Based on a 2D array detector (digital camera), the imaging speed of QPM is only limited by the speed of detector (from tens to thousands of Hertz) which is well-suited for real-time monitoring of biological events. Unlike traditional workhorse technologies such as epifluorescence microscopy and phase contrast microscopy, QPM faces some limitations which need to be overcome before it can become an universal imaging tool. Primary limitations of QPM are the inability to distinguish heterogeneity along the vertical direction, and additional digital reconstructions. For full axial resolvable QPM, one needs to use optical tomography techniques²³⁻²⁴. However, optical tomography is laden with multi-frame acquisitions and additional digital transforms²⁵ that contribute to a reduction in imaging speed and is precluded from its applications on real-time image reconstruction. On the other hand, automation of the QPM reconstruction processes is in great demand to make it accessible for biologists to obtain statistically meaningful data.

Understanding dynamic processes and mechanisms of disease-related events is important in biology studies. The blood disease and disorders are one of the diseases that remain to be overcome due to complexity in fluidic flows within living vessels²⁶⁻²⁷. Red blood cells (RBCs) and platelets contribute to majority of the blood components. The diseases related to morbidity of RBCs and platelet disorders lead to the major lethality, such as malaria²⁸, haemophilia²⁹, and thrombosis²⁷. A number of research tools and methods have been developed in the past two decades for investigating the fundamental mechanism of blood related disorders with dynamic manipulation and cellular response measurement³⁰⁻³¹. Microfluidic technique is one of the tools that can realize high-throughput measurements on single-cell biophysical properties at a relatively low cost³²⁻³⁷.

Thus, this thesis is primarily motivated to develop an automatic volumetric imaging system to investigate the dynamic response of RBCs and platelets under fluid shear conditions. For RBCs, the focus on parasite-induced alteration in morphology and deformability is motivated by efforts in diagnosing early stages of malaria infection. Study of platelets provides an effective real time measurement for investigating the platelet disorders and thrombosis. Dynamic imaging of platelets aggregation and thrombus formation in microfluidic device has been ongoing for more than ten years³⁸. However, most quantifications of

platelet and thrombus formation are accomplished by integrating the fluorescence intensity of images. The morphological characterization of individual thrombus received less attention. One of the main reasons is that the fluorescence imaging techniques usually obtain morphology reconstruction at the cost of sacrificing the imaging speed by vertical laser scanning. However, imaging speed is vital in investigating dynamic events, for example, thrombus formation and embolism in the fluidic. As a result, real-time morphological measurements of thrombus formation and embolism is targeted using QPM system developed in the thesis, which could potentially provide the ability of phenotypic quantifications and therapy tests. Since every single imaging technique has its own merit and shortcoming, the combination of two or more imaging modalities have been studied and employed to provide multifunctional imaging³⁹⁻⁴¹. At the end of the thesis, an initial version of a multimodality imaging system was designed and implemented for microfluidic experiments.

1.2 Specific aims

In this thesis, the target is to develop an advanced volumetric imaging system for efficient investigation on the dynamic biological events related to vascular diseases using microfluidics.

Aim 1: Develop an adaptable QPM system for rapid imaging with graphical user interface and basic data analysis functions.

Phase contrast microscope⁴² has been developed for the last century and still is a key imaging modality in all biology laboratories. It provides high contrast images of nearly transparent biological samples by converting the phase differences ($\Delta\phi$) to intensity difference (ΔI) in the images. This conversion remains qualitative, not quantitative. However, QPM technique⁴³⁻⁴⁴ allows the phase information of the sample to be retrieved quantitatively, which has emerged as a powerful imaging tool for label-free biological studies. Off-axis digital holographic microscopy (DHM)¹⁹ is one of the typical interference methods of QPM, which can be easily implemented and adopted in other microscopy modality and is capable to retrieve the phase map from a single image. Thus, high speed imaging can be achieved using a fast camera as a recording device. However, the phase reconstruction procedure of DHM usually requires specialised knowledge in optics and image

processing. DHM is well-equipped to record micrometre size-scale dynamics in optically thin specimens but provides poor optical resolution for denser biological sample owing to multiple light scattering that severely distorts the holograms. These two vulnerabilities limit the usage of DHM in biology studies. Therefore, the aim of the first part of the thesis is to develop an automated DHM system that can be operated in a variety of conditions through improvement in the phase reconstruction/retrieval procedure, equipped with a graphic interface and basic cell extraction and analysis. The detailed work is described in chapter 5.

Aim 2: Microfluidic adhesion assay for RBCs morphology and deformability investigation using QPM system.

Malaria ⁴⁵ related deaths are often linked to changes of morphological and biochemical properties in RBCs as the result of malaria parasite invasion, which severely impairs functions of RBCs in blood flow. While it is difficult to measure RBC function in living blood vessels, it is routinely possible to make use of microfluidic technology to mimic the flow of blood. The aim of this part is to apply the proposed DHM system, combined with the microfluidic tool, to generate an efficient investigating method for dynamic morphological changes of the RBCs. We further developed simple adhesion assay in microfluidic devices to create shear stress on the cells. The healthy RBCs, artificially stiffened RBCs, and the malaria parasite-infected RBCs are examined under fluidic force and automated DHM so as to provide a comprehensive investigation.

Aim 3: Direct mapping thrombus dynamic in microfluidic with QPM system.

Blood coagulation and thrombus dynamic events are known to be dominated by platelet function ⁴⁶. Non-invasive volumetric quantification of thrombosis has potential to gain insight into functional response of platelet aggregates under flow and possibly lead to early diagnosis of thrombosis diseases. Using the QPM system developed in chapter 5, an efficient label-free quantification method for thrombus formation and embolism under shear condition has been achieved. The target is to make full use of DHM system to study thrombosis process by providing comprehensive static and dynamic measurements on thrombus formation and embolism events.

Aim 4: A multimodality imaging system for microfluidic application to

provide both molecular and morphological information.

Multimodality imaging solutions have gradually become popular in field of microscopy because of their ability to generate multiple-parameter measurements of a single sample at the same time ⁴⁷. Majority of the existing DHM are typically combined with standard epi-fluorescence microscope. The final work of the thesis aims to develop a multimodality volumetric imaging system to provide both molecular and morphological information of biological samples in microfluidics. The inverted selective plane illumination microscopy (iSPIM) is chosen to be integrated with DHM to supply the fluorescent signal, as it is a novel fast three-dimensional fluorescent image solution that can be simply added on to a conventional inverted microscope. Aside from demonstrating the development of integrated DHM with iSPIM (H⁺iSPIM) system, the goal of this part of work includes the development of practical solutions to implement multimodality imaging in microfluidic environment using (H⁺iSPIM) system, by analysing the optical aberrations under different sample mounting conditions.

1.3 Thesis outline

This section explains the layout of the thesis.

In chapter 2, the current development of volumetric microscopy techniques and the review of current volumetric imaging techniques are included. In the third chapter, the biomechanics of anucleate blood cells, red blood cells and platelets, are introduced. In chapter 4, the basic of microfluidic techniques is discussed, along with the device fabrication used in the thesis.

Chapter 5 elaborates the technical progress made in development of the automated DHM system. Chapter 6 and 7 then focus on the applications of the DHM in red blood cells and platelet/thrombus application respectively. Chapter 8 covers the development of multimodality imaging system for microfluidic applications.

The last chapter concludes the findings in the thesis and discusses the challenges and strategies for improving the proposed imaging system and the future perspective of the volumetric imaging technique in bio-applications.

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Chapter 2 Optical volumetric microscopy

2.1 Microscope from 2D to 4D

The very first light microscopy was made by two Dutch spectacle makers, Zacharias Jansen and his father Hans in the late 16th century ¹⁻². It was a simple design consisted of several lenses of which the position can be adjusted in a tube. After that, the development of microscopy made possible the discovery of cells (by Robert Hooke and Anton Van Leeuwenhoek in 17th century ³), which started the journey of microbiology by providing the ability to observe the small structure that had never been seen by human naked eyes before. After that, the innovations in microscopy field have been directly driven by the needs of cell biologists. Phase contrast microscopy has been invented in the early 1930s and provided high-contrast images for thin, transparent biological samples. Later the discovery of fluorophore ⁴ and the invention of fluorescence microscopes ⁵ launched the field of molecular biology by offering the ability to map out specific proteins in biological specimens. Since then, the progress in the technical development and fluorescent probe synthesis has contributed to the continuing widespread utility of the fluorescence microscope and allowed the biology to undergo a renaissance in the last decade.

Living organisms such as bacteria, cells, and organs are highly dynamic and complex. With the progress of biological studies and the microscopy techniques, biologists want to observe the biology specimens full view with three dimensions as well as to record the full biological process like metabolism, movement, interaction, and growth. Therefore, ability to observe and measure the subjects in four dimensions (4D), three-dimensional (3D) space (x, y, z) and time (t), becomes more and more imperative in optical microscopy field. In other words, the spatial-temporal resolution of the microscopy system becomes a significant criteria ⁶. 3D reconstruction of an investigated specimen is highly desirable for a better understanding of its structures in almost all biological studies. The ability to take time-lapse 3D images or high frame-rate 3D videos of the specimen is superior since it is able to provide a 3D view of dynamic responses

and interactions ⁷⁻⁸. That is highly valuable for understanding fundamental mechanisms of cell functions and pathological processes.

To achieve 4D microscopy, a volumetric microscopy system for dynamic cells studies is developed and applied in this thesis. In the following chapter, a review of the current approaches to volumetric microscopy is provided based on laser scanning and coherence imaging.

2.2 Approaches to volumetric imaging

In this chapter, a review of current optical volumetric microscopy techniques is provided as the background and introduction of the thesis from imaging system perspective. The current approaches are categorized into three main types; (1) Laser Scanning Microscopy, (2) Quantitative Phase Microscopy, (3) Multimodality 3D imaging solution.

2.2.1 Laser Scanning Microscopy: point, sheet and multi-point

Laser Scanning Microscopy (LSM) ⁹⁻¹¹ is a type of microscope that use a laser as the light source with different illuminating methods or different excitation mechanisms to create and collect the fluorescent signals from a plane or from a point within a labelled sample. The excited plane or the point is selected and scanned throughout the whole sample in three-dimensional space and eventually can be mapped out to reach a volumetric image.

2.2.1.1 Background

There are three key techniques paving the path for the birth of laser scanning microscopy; the invention of Laser, the discovery and synthesis of fluorescence dye, and the production of photodetectors.

The key properties of the laser light are the narrow band of wavelengths (monochromaticity), spatial and temporal coherence ¹²⁻¹⁵ and the low divergence directional beam output ¹⁶⁻¹⁸. The ground-breaking theoretical work on the operating principles of lasers ¹⁹ was conceived by Charles Hard Townes and Arthur Leonard Schawlow. Around ten years later, the first laser device, a pulsed ruby laser, was made by Theodore H. Maiman at Hughes Research Laboratories in 1960 ²⁰. The inception of the first confocal laser scanning microscopy came into being in 1969 ²¹. the discovery of fluorescence phenomenon which dated

back to 1500s. However, it was not until the 1800s that George Gabriel Stokes⁴ used a prism to disperse the sunlight to illuminate a solution of quinine and found out that there was no effect until the solution was placed in the ultraviolet region of the spectrum. Stokes proclaimed that fluorescence is of longer wavelength than the exciting light, the wavelength shift is now known as Stokes Shift. But this experimental observation was only formalised in 1935 by a Polish physicist named Aleksander Jablonski. He formally developed a schematic that describes the theoretical basis for fluorescence excitation by illustrating the transitions of energy levels of molecular absorbance and emission of light²². These schematics are referred to as Jablonski Energy Diagrams. Figure 2.1 shows the simplified Jablonski Energy diagram of the one-photon, two-photon, and three-photon excitation. Discrete electronic (energy) states are indicated by horizontal lines. The colored waves are represented for excitation light and emission light in the visible band (provide indicative wavelengths). In laser fluorescence microscope, the excitation light source refers to the laser source and the emitted light refers to the fluorescence signal measured by photodetector which is then digitally encoded (map pixels to position) to create the final fluorescence images.

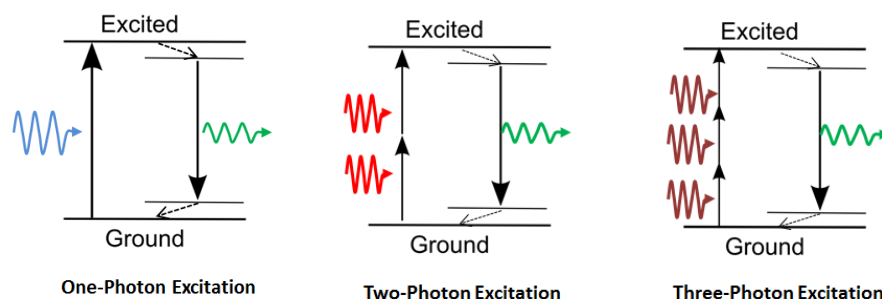


Figure 2.1 Simplified Jablonski Energy diagram of the one-photon, two-photon and three-photon excitation. Blue wave indicates excitation light of 360 nm wavelength. Red waves indicate excitation light of 720 nm wavelength. Brown waves indicate excitation light of 1080 nm. Green waves indicate emission light of 460 nm wavelength.

Figure.2.1 illustrates the loss of energy during transitions. This meant that fluorescence light would typically have weaker energy along with the change in wavelength compared with excitation light. In order to efficiently measure the fluorescence signal, advances in photo-electronic coupling devices are made. It is combination of photochemistry and optoelectronics that gave birth to the modern LSM technology. Based on different excitation mechanisms, different types of

laser scanning microscope (confocal, two-photon, and three-photon) have been developed, which are introduced by following sections.

2.2.1.2 Point scanning

Confocal LSM²³⁻²⁵ and multiphoton LSM^{9, 26-28} visualize the sample by focusing the laser beam to a single, diffraction-limited point to excite the tiny region ($1.22 \lambda/NA$) within the fluorescently-labelled sample. The focus spot is raster scanned over sample plane to create a 2 D image. Repetitive raster scanning at incremental vertical steps creates the stacks of plane images and maps out the whole bulk sample in three-dimensional space. The size of beam waist typically determines lateral resolution. The ability to visualize a thin transverse plane within a bulk sample that is several orders of magnitude thicker than axial focus of the beam is called optical sectioning. In confocal LSM, optical section ability comes from an aperture that is implemented to physically block the signals generated outside of the optical focus. For the multiphoton LSM, the laser beam does not generate any appreciable emission signal outside of the focal plane. Since this non-linear quantum mechanical process is possible with a square of excitation intensity. Only a single diffraction limited point in the focus gathers enough photon density for triggering multi-photon excitation. Longer wavelengths laser source used in multiphoton LS offers deeper penetration in thick tissue and also reduce detrimental effects such as autofluorescence and photodamage. Figure 2.2 shows the schematic diagram of optical section and scanning mechanism in confocal and multiphoton microscopy.

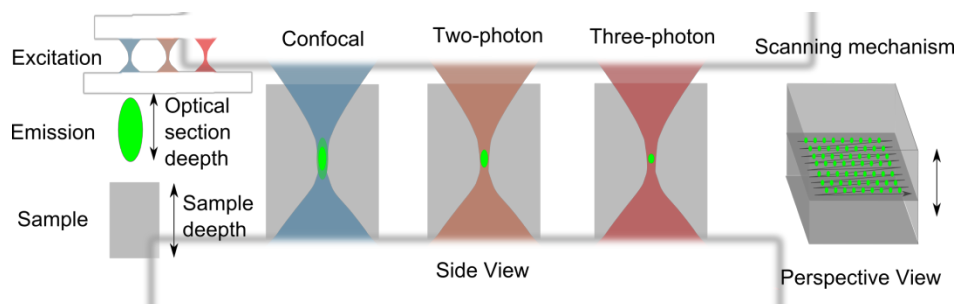


Figure 2.2 Optical section and scanning mechanism for confocal LSM and multiphoton LSM.

To improve the fourth imaging dimension, time, there are two main considerations to address. One is the temporal resolution and the other is the sample viability. The former mainly depends on the mechanical scanning speed

and the trade-off between the image resolution as well as the size of volume acquisition. For thicker samples of up to hundreds of micrometres, the number of scanning points needs to increase so as to reach the full imaging depth. However, such large volumetric stacks slow down the overall imaging speed (several seconds for galvanometer mirror scanners) and consequently limits the ability to capture fast temporal events (milliseconds). As such, LSMs are generally inefficient for observing the millisecond dynamics within the entire imaging volume. However, it is more useful for probing selected regions of interest within the entire sample, due to that the position of the focal point can be accurately determined and controlled, and no out-of-focus area affects the imaging. In addition, the penetration depth of the multiphoton LSM can reach millimetre range owing to the use of longer wavelength laser source, of which the optical scattering coefficient is lower. As for sample viability, photobleaching²⁹⁻³¹ and photodamage³² are two detrimental issues of fluorescence microscopy in the study of living cells, tissues or organisms³³. Bleaching is irreversible decomposition of the fluorescent molecules because of oxidation process. Photodamage is the dissociation of fluorescent molecules which is a highly destructive process. Those problems restrict fluorescence microscope for live cells long term imaging experiments, especially the confocal LSM and two-photon microscopy. The three-photon LSM decreases photobleaching by using longer wavelength and extremely short dwell times (~50 ns). The proper choice of image acquisition parameters, optimization of filter sets and hardware synchronization could also reduce the risk of photobleaching and photodamage³⁴.

In all, the point scanning methods provide four-dimensional (x, y, z and time) reconstruction of fluorescently labelled the sample with high level of molecular specificity using wavelength (λ). This can be refer to as the fifth imaging dimension (colour/wavelength coding). However, the performance in each dimension is mutually constraint. In microscopy, it is long known that the improvement in the performance in any of these dimensions can only be achieved by sacrificing another dimension. For example, larger field of view usually requires longer imaging time or lower image resolution..

2.2.1.3 Plane scanning

Light sheet fluorescence microscopy (LSFM)³⁵⁻³⁹, also known as selective/single plane illumination microscopy (SPIM), uses a sheet of light to illuminate the thin plane within a sample and collects the fluorescence signal from the direction orthogonal to the sheet by a camera instead of photodetector [Figure 2.3.a)]. The innovation behind LSFM is the orthogonal placement of the imaging lens and scanning sheet of light that illuminates the sample. This simple reconfiguration of the scanning path lifted the restrictions of conventional LSM limitations whilst maintaining high cellular imaging resolution ($0.61\lambda/NA$) with low phototoxicity over an extended volume (hundreds of microns to millimetre scale). Figure 2.3.b) shows the different methods for creating the light sheet. In the simplest configuration, a cylindrical lens can be used to shape the laser beam to a static light sheet. Another method is to simply scan a weakly focused Gaussian beam along in one direction to create a light sheet. This is called digital scanned laser light-sheet fluorescence microscopy (DSLM)^{37, 40}. A Bessel beam can be used to create a thinner and longer focused central core of the beam⁴¹. However, a series of higher order concentric rings of the Bessel beam where majority of the optical power resides reduces sample viability and increase out of focus fluorescence. To circumvent that, two-photon variant of Bessel beam light sheet has been used to achieve a thinner and longer sheet for better image quality and larger field of view⁴².

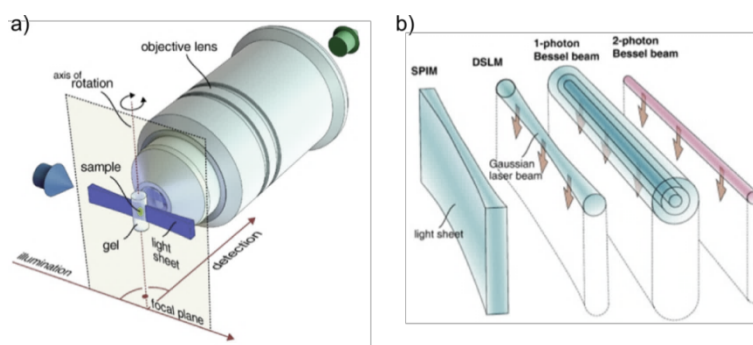


Figure 2.3 Concept of light sheet fluorescence microscopy operating principle. The figure is reprinted, with permission, from Weber et al. 2011⁴³, © 2011 Elsevier Ltd.) (a) A focused sheet of light is projected to illuminate the thin plane within a sample. The fluorescence signal is collected by an objective lens from the direction orthogonal to the sheet. The sample is mounted in a round tube and fixed in agarose gel. (b) Commonly used methods of generating the light sheet.

Chapter 8 will provide additional discussion on the different variation of the LSFM configurations. In all, the LSFM family has been adapted to different biology applications and are able to address biological questions that cannot be considered before ^{7, 44}, such as recording nuclei localization and movement in entire zebrafish embryos for long terms ³⁷. Although there are drawbacks and unsolved aberration and sample preparation challenges (discussed in chapter 8), in general, the light sheet technique is a great choice for four-dimensional observation of cells, organs and embryos due to the faster imaging and less photodamage compared with previous point scanning laser microscopy techniques.

2.2.1.4 Multi-point illumination

Although the plane scanning method greatly increases the performance of temporal resolution and the specimen lifespan, high spatial resolution and high temporal resolution are still unachievable at the same time. One solution is to illuminate multiply points on the sample simultaneously and collect light from all of them at the same time. The technique is also called structured illumination and is used in other non-labelled microscopes as well. Here two of most common multi-point illumination techniques are introduced in the concept of LSM, lattice light sheet microscopy and the spinning disk confocal microscopy.

Lattice light-sheet microscopy ⁴⁵ comes out as a superior solution based on the light sheet microscopy configuration. Optical lattices are periodic interference patterns in three dimensions created by multiple plane waves propagating in well-defined directions in space [second images in figure 2.4 c and d]. This technique has been adopted in atom cooling or trapping applications ⁴⁶ as well as in nanolithographic fabrication approach ⁴⁷ for decades. To apply optical lattices in light sheet microscopy, a fast switching spatial light modulator (SLM) is placed at the conjugate plane of the sample, before an annular mask. The diffracted incident laser light after SLM is filtered by the mask and then focused by the excitation objective to produce a lattice light sheet. Figure 2.4 demonstrates the configuration and the performance of the lattice light sheet work from Chen et al. 2014 ⁴⁵. Lattice light-sheet microscopy greatly reduces photobleaching and phototoxicity by reducing the peak intensity of the light sheet. At the same time, it achieves the sub-second interval imaging speed with desirable axial resolution

(280 nm), which cannot be reached by other fluorescence microscopes. Moreover, the negligible background enables the technique to study the dynamic activity in more dense specimens or more complex environments. However, lattice light sheet microscopy also suffers from sample-induced aberrations with the increasing imaging depth. To compensate for these aberrations, imaging beyond certain depth will require adaptive optics in both excitation and detection pathways⁴⁸. Meanwhile, it is necessary to carefully choose whether the fast-subcellular dynamics (seconds and minute) events or the full course of longer development (hours or even days) to be observed. Since the system setting needs to be optimised for specific temporal resolution.

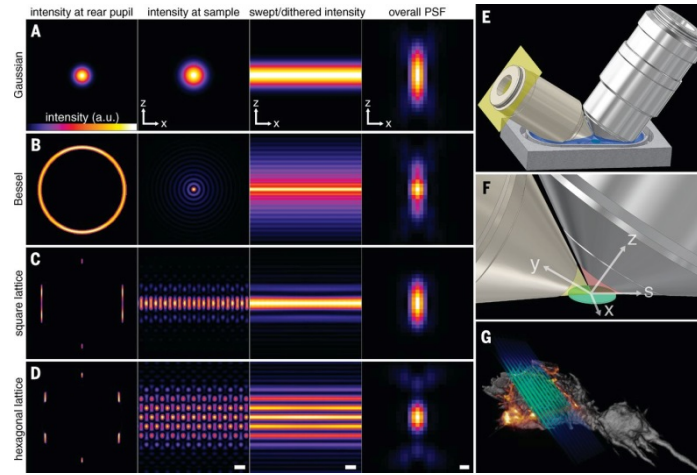


Figure 2.4 Methods of lattice light-sheet microscopy, reprinted with permission, from Chen et al. 2014⁴⁵ © 2014, American Association for the Advancement of Science. (A) The traditional approach, DLSM. (B) One-photon Bessel beam DLSM. (C and D) The produced optical lattices create periodic intensity patterns of high modulation depth across the plane. The square lattice (C) optimizes the confinement of the excitation to the central plane, and the hexagonal lattice (D) optimizes the axial resolution as defined by the overall PSF of the microscope. The scale bar is 1 μm for the second and the third columns and 200 nm for the forth columns. (E) and (F) The core of lattice light-sheet microscopy with orthogonal excitation (left) and detection objectives (right) that are both dipped in a media-filled bath. (G) Representation of a lattice light sheet (blue-green) intersecting a specimen (grey) to excite fluorescence (orange) signal.

Another multi-point illumination technique for both high spatial resolution and temporal resolution is spinning-disc confocal microscopy⁴⁹⁻⁵³. The design and improvement of the spinning-disc confocal microscopy started from 1967 when David Egger and Mojmír Petráň modified the Nipkow disk to a disk with

multiple rounds of spirally placed holes and implemented it on a reflected-light microscopy⁵⁴⁻⁵⁵ to achieve better resolution by blocking the stray light. The designed system was called Tandem-Scanning Reflected-Light Microscope. This ingenious operation pioneered the modern spinning disk confocal microscopy, in which the spinning disk is used to create fast scanning multi-point illumination on the sample. Compared with single point scanning method in confocal LSM, this multi-point illumination increases the imaging speed [360 fps with 10*7 mm FOV (basic model CSU-X1, YOKOGAWA Spinning Disk Field Scanning Confocal Systems)] and lowers laser radiation damage. The imaging speed can Figure 2.5 shows an example of a spinning disk confocal microscopy. The detailed configuration can refer to reference [57]. However, there are several limitations. The first is called pinhole crosstalk⁵⁶. It happens when imaging the thick sample where the out-of-focus fluorescence emission can scatter pass through adjacent pinholes, eventually increasing the background noise. Another disadvantage of the spinning-disk confocal microscopy is the relatively inefficient use of the excitation light. The total power of the laser is decentralized to multi-points, which inherently hampers very dim fluorescent specimen observation.

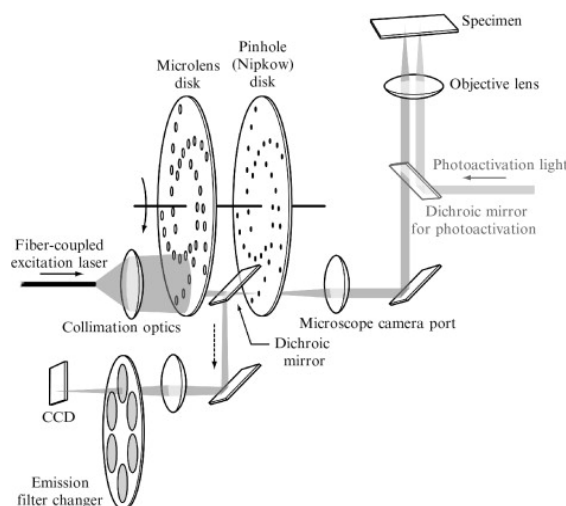


Figure 2.5 Schematic layout of a Spinning Disk Confocal Microscopy, reprinted with permission, from Stehbens et al. 2012⁵⁷ © 2012 Elsevier.

In all, the light sheet and the multi-points illumination with array detector creates the two-dimensional images in a much more efficient manner than the point scanning. However, each technique has its own merits and demerits. In the next part of the chapter, quantitative phase microscopy is introduced, which

provides higher imaging dimension (depth) from 2D images through optical interference and/or diffraction of the light.

2.2.2 Quantitative phase microscopy: interference and diffraction

Apart from fluorescence excitation with the scanning mechanism, which is based on the energy of photons, the wave nature of the illumination light could also be a solution for volume reconstruction. In this section, the coherence property of the light wave is introduced first, followed with quantitative phase microscopy techniques for volumetric imaging. The technique does not require sample to be stained or labelled, and the volume reconstruction can be achieved without raster scanning mechanism.

2.2.2.1 Background: interference and diffraction

From 19th century till today, optical diffraction and interference has lay the foundation of many physical and engineering sciences. The famous experiment that led to this was conducted by Sir Thomas Young in 1801, who publicly demonstrated the double-slit interference effect ⁵⁸. It led to the understanding of how light propagate over distance, which now can be easily computed using modern Fourier Optics ⁵⁹.

Diffraction refers to the effect when light waves encounter an obstacle or a slit, where the wave front will get slightly bent. The amount of bending is negligible if the wavelength of the light is negligible compared with the size of the slit. However, if the wavelength and the size of the slit/obstacle are similar, the amount of bending is considerable and can be easily observed. While interference is a phenomenon that two waves are superposing to form a resultant wave, an interference pattern is the intensity of the resultant wave that is observed. The source of two light waves must be coherent, which means they maintain a constant phase (ϕ) with each other and they are monochromatic, of the same frequency (f). Figure 2.6 shows a schematic drawing of an example of diffraction and interference. In the figure, the light wave (plane wave) is diffracted by three obstacles (or two slits). And two beat waves after the obstacles interfere with each other.

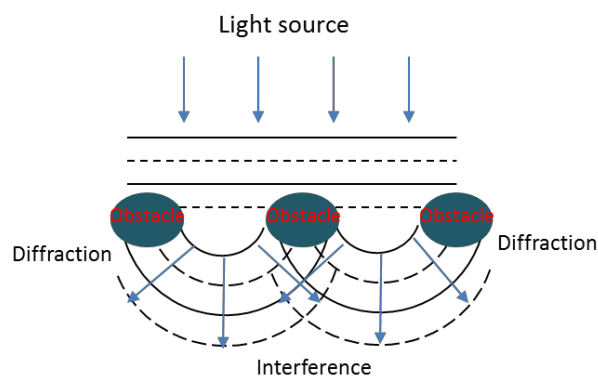


Figure 2.6 Schematic drawing of an example of diffraction and interference. The blue lines with arrows represent the propagation direction of light. The black solid lines represent the positive amplitude of the light waves, while dash lines represent the negative amplitude. Diffraction occurs where light meet obstacles/slits. Interference occurs when two beat waves overlap.

Interferometry is one of the most widely used metrology techniques by means of interference phenomenon for subtle displacements, refractive index changes and surface irregularities measurements. It has been applied to a great range of physical sciences and engineering like astronomy, optical metrology, fibre optics, seismology, spectroscopy remote sensing, biomolecular interactions, surface profiling, microfluidics and velocimetry ⁶⁰. Also, it is the cornerstone of the interference based quantitative phase microscopy. As for diffraction, the X-ray diffraction crystallography is one of the most successful utilizations of diffraction phenomenon to explore the molecular structures at angstrom. In optical microscopy field, Ptychography ⁶¹⁻⁶² was introduced (first in electron microscopy but immediately merged into optical microscopy) as a representative of the diffractive imaging method. It employs the multi-angle illumination and measures every diffraction pattern at each angle to reconstruct a single image of the sample by iterative algorithms. Diffraction phase microscopy ⁶³⁻⁶⁴ is another optical microscopy technique to reconstruct the sample structure by implementation of physical grating or some phase modulator to create the diffraction pattern. More recently, the use of near-field interferometry has been used to quantify molecular weight of single protein. ⁶⁵ As compared to LSM technologies, interferometry based measurement tools can easily reach molecular resolution.

2.2.2.2 Phase microscopy from qualitative to quantitative

In this section, applications of interferometry in biological imaging are focused. In 1934, phase contrast microscopy⁶⁶ was proposed by Dutch physicist Frits Zernike who was awarded the Nobel Prize in Physics in 1953. The ability to provide a high contrast of fine structures of transparent thin biology specimens led to widespread use of phase contrast microscopy. Since then there are several other types of imaging contrast techniques such as differential interference contrast (DIC) microscopy and Hoffman contrast⁶⁷. It is also widely used to enhance the contrast in unstained, transparent samples. Both techniques are using interference of two portions of light to convert phase gradients in the sample into the intensity difference that is rendered in the final image plane, thus making the transparent sample obvious. However, these two time-honored phase microscopy techniques remain qualitative not quantitative. The intensity difference (ΔI) represented is coupled nonlinearly and only increases the contrast but does not offer the specific phase difference ($\Delta\phi$) or saying optical path difference ($n*d$, n for refractive index of the medium and d for distance that light travels).

It was in the 1940s that Gabor proposed holography⁶⁸, a method to record both amplitude and phases in one photograph. The word holography was from two Greek words, “holos” and “grafe”, which means “entire” and “writing”. The term was created to indicate that the entire information of the light field is recorded. In Gabor’s original method, the object was placed after the focused point of a coherent source. A photographic plate was placed at the far field to record the interference between the light diffracted by the object and the collinear background. Later, photography was illuminated by the same copy of the light source to produce the image of the object. The method was proposed for improving the resolving power of electron microscopy initially. After around 20 years, in the early 1960s, the development of lasers suddenly changed the situation. The method was applied in optical microscopy and paves the way for the invention of quantitative phase microscopy (QPM). Now QPM is a collective name for a group of microscopies with the ability to quantitatively measure the phase delay ($\Delta\phi=2\pi* \Delta n/(\lambda*h)$, Δn for refractive index difference between a sample and around medium, λ for wavelength of the light, h for thickness of the sample) introduced by biological sample without any staining can reveal the

veritable thickness of the sample (with known refractive index of the sample and surrounding medium) ⁶⁹⁻⁷⁰. Thus, the morphology of the sample can be retrieved for biological investigation of structure and dynamics.

QPM can be divided into two main groups: off-axis method and common-path method. The off-axis QPM ⁷¹ requires separated two copies of light to create the interference pattern just like an interferometer. One of two arms of light passing through the sample gets delayed. The delayed phase is encoded in the final interference pattern, which is also called hologram or holographic image. The setup of the systems is usually similar to various interferometers. Off-axis digital holographic microscopy (DHM) ⁷²⁻⁷³, and Hilbert phase microscopy ⁷⁴⁻⁷⁵. The common-path method does not introduce the second arm of light to produce the interference. It acquires the phase information by introducing diffractive elements. Figure 2.7 shows two examples of common-path method techniques. Diffraction phase microscopy ⁶³⁻⁶⁴ uses a grating at the image plane of the microscopic system to create the diffraction pattern that contains the full spatial information of the sample. Fourier Phase microscopy introduces a programmable phase modulator as a spatially controllable phase mask in the conjugate plane of the image to generate the phase shift between scattered and unscattered light from the sample (unscattered light, the fine dash line in Figure 2.7 (b), is focused at the center of the optical axis in the conjugate plane). It resembles the phase mask in phase contrast microscopy. The phase difference can be calculated by modulations of four successive $\pi/2$ increments phase shift. Therefore, it requires at least four holograms to reconstruct one phase image. Among these different configurations of QPM, some aim at the higher contrast of phase images while others focus on improving temporal sensitivity. The most important factor in the dynamic volumetric imaging concept is the acquisition rate. Therefore, the QPM techniques that need multiple holograms reconstruction will not be considered for dynamic biological studies. The single shot benefit of the off-axis digital holographic microscopy is therefore making it a fast imaging tool that is suitable dynamic biological process i.e. blood cell under fluidic flow, which is the main biological process being studied in this thesis. Detailed introduction and implementation of this imaging tool are further presented in chapter 5.

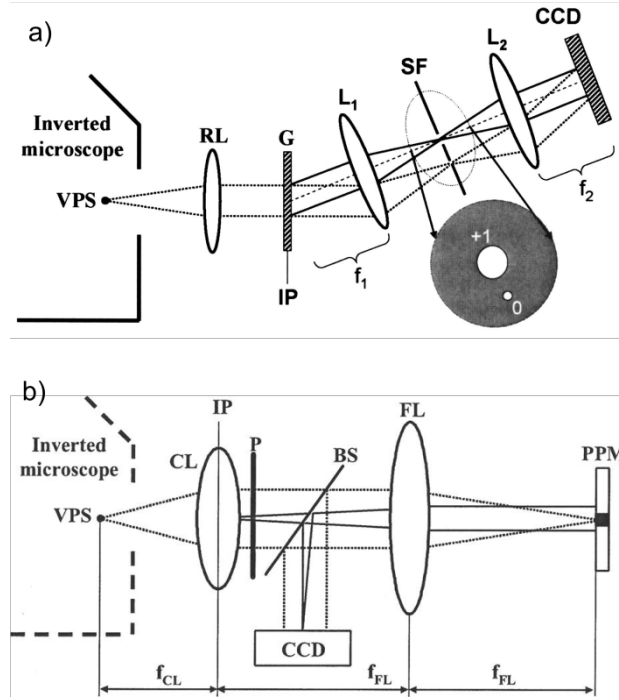


Figure 2.7 Quantitative phase microscopy. a) The diffraction phase microscopy configuration from Popescu et.al⁶⁴ with permission © 2006 Optical Society of America. A relay lens (RL) is placed at one focal length after the virtual source point (VPS). Grating (G) is at image plane (IP). A 4-f system with $L_{1,2}$ lenses with respective focal distances $f_{1,2}$, is employed after the grating. And a spatial filter (SF, expanded in the inset) is at one focal length (f_1) after L_1 . b) Fourier phase microscopy configuration⁷⁶ with permission © 2004 Optical Society of America. Correcting lens (CL) is positioned at IP. A Polarizer (P) is placed after the CL. A Fourier lens FL focuses the unscattered light into the centre of the programmable phase modulator PPM. The light is divided into two copies by the beam splitter (BS) and one arm of light was spatially modulated by the PPM. Two arms of light are recombining at the CCD by the same BS.

A main bottleneck in QPM is the optical path and reconstruct the thickness of the biological sample without scanning through it. The reconstruction renders the two and a half dimension information of the sample, which is similar with topographical imaging but not the same with 3D reconstruction from the laser scanning techniques. Figure 2.8 shows an example of a red blood cell volumetric image from an off-axis digital holographic microscopy. Under some circumstances, the two and a half dimensional (2.5D) image is sufficient to investigate the biological relevance, such as adherent cells morphology study. Here we shall define 2.5D image is a height/phase delay map, for example, Figure 2.8, in which one and only one value in z coordinate represents the height/phase

delay of the sample. Therefore, choosing the application carefully is required to fully make use of the volumetric mapping ability of the QPM in biological study.

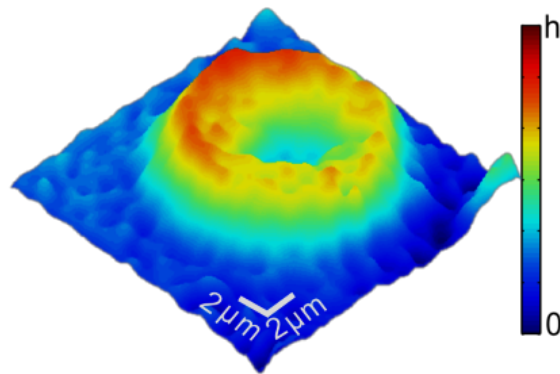


Figure 2.8 A volumetric image from off-axis digital holographic microscopy.
The colour map represents the thickness ($h = 2.5\mu\text{m}$)

Nevertheless, there are still other solutions to acquire the full three-dimensional images in QPM techniques. Tomographic phase microscopy⁷⁷ for example uses a phase-shifting laser interferometric microscope (an off-axis QPM technique) with variable illumination angle to offer the full three-dimensional reconstruction of the sample.⁷⁸ Figure 2.9 demonstrates the setup and the three-dimensional reconstruction of a HeLa cell from Choi et.al⁷⁷. The multiple angles of illumination is achieved by a galvanometer-mounted tilting mirror (GM) scanning. Two acousto-optic modulators (AOM) are equipped in the reference arm to produce the frequency shift of the laser beam by 1,250 Hz. Therefore, the work represented the time-dependent changes of the sample in three minutes' interval. Since the four frames required for one angle illumination data, and 81 angles (-60 to +60 degrees in steps of 1.5 degrees), large amount of data is required to reconstruct a single three-dimensional image. Therefore, the technique is also facing the trade-off between spatial and temporal resolution just like the laser scanning microscopy.

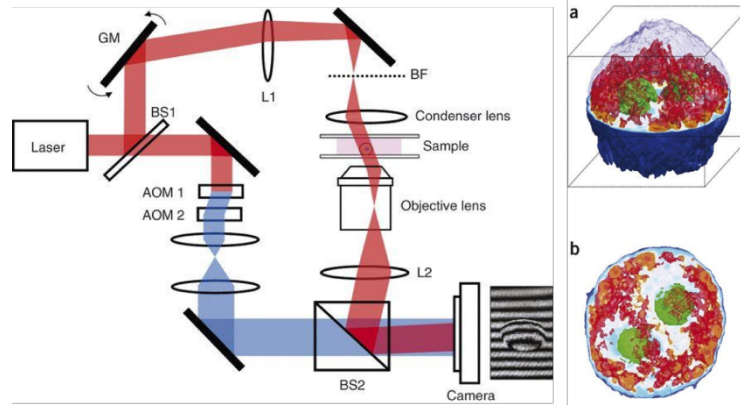


Figure 2.9 Tomographic phase microscopy system, reprinted with permission from Choi et.al ⁷⁷ © 2007, Springer Nature. a) and b) are perspective view and cross section top view of a HeLa cell imaged by the system.

In all QPM provides the ability to achieve the limited volumetric imaging (two and a half dimensions in this significance) for highly dynamic events with CCD camera technique and off-axis methods. Sample can be unstained and is free from the photo-damage, which extends the observation time for investigating the events of interest. Also, the technique could make use of scanning and rotating the sample to achieve true three-dimensional images by sacrificing the imaging speed to some extent.

2.2.3 Multimodality volumetric imaging techniques

In the previous sections, two main kinds of volumetric imaging method are discussed. Each technique has its own merits and demerits. Confocal LSM and multiphoton LSM are based on a single focused point of the high-power laser beam to excite the fluorophore and photon detectors (PMT or APD) to identify the fluorescence signal. The control and the penetrability of the laser beam, as well as the high sensitivity of the photon detector, make the technique great for deep tissue imaging ⁷⁹⁻⁸⁰ and experiments of laser-induced injury for live animals. For example the *in vivo* brain imaging ⁸¹⁻⁸² for neuroscience studies or localized photomanipulation experiment ⁸³⁻⁸⁴ for observing the response to local injury or investigating the photobleaching recovery kinetics ⁸⁵. The light sheet illumination and the multi-point illumination techniques accelerate the image speed and reduce the phototoxicity by utilizing the plane illumination, which extends the sample duration and broaden the applicable biological studies. However, the light sheet configuration requires unconventional sample mounting. Spinning disk confocal

microscopy sacrifices the efficiency of the illumination power. The QPM technique, as another volumetric imaging solution, offers the non-invasive volume information of the sample at high frame rates (camera-based) which is powerful for dynamic cytomorphology research, whereas it could not provide the single protein specificity that fluorescence microscopy does. Table 2.1 provides a summary of main microscope techniques discussed in this chapter.

Microscope Technique	CLSM	Multi-photon LSM	Light sheet Microscope	Spinning Disk CLSM	DHM
Image speed *	0.5-10 fps (512 *512 pixel)		10-30 fps 1000*1000 pixel	Up to 200 fps Full-size image	Up to 500 fps (1280*1080 pixel)
Photobleaching/ phototoxicity	Severe	Limited	Low	Low	None
Primary use (in biological studies)	2D imaging (optical sectioning)	In-vivo 2D/3D animal imaging, depth tissue imaging	3D/4D imaging for entire organ/ embryo	Live cell 4D imaging	Live cell 4D imaging, live cell mass monitoring

Table 2. 1 Summary of different microscope modalities. * Image speed (frame rate) referred to commercial microscopes (models: Nikon C2+ confocal microscope; Nikon A1 MP+ Multiphoton microscope; ZEISS Lightsheet Z.1 microscope; CSU-W1, YOKOGAWA Spinning Disk Field Scanning Confocal Systems; iX Cameras i-speed 210/211)

As every single technique has its own forte and limitation, it is wise to develop the hybrid multimodality imaging system to compensate for the vulnerabilities. For instance, Optical coherence tomography, a phase imaging technique, has been benefited from combining with other imaging techniques so that it can provide sensitivity and specificity⁸⁶⁻⁸⁸. Fluorescence image was used to

guide the OCT scanner to enhance early cancer detection in rat bladders⁸⁹. A functional-OCT system was incorporated with two-photon-excited fluorescence microscopy⁸⁷ and a confocal fluorescence microscopy was added into the OCT system⁹⁰. Recently, Yi et al. combined OCT with oblique scanning laser microscopy to provide fast volumetric structural and molecular images⁸⁸, which is a great tool for tissue studies. Other examples of hybrid imaging techniques are combinations of quantitative phase microscopy with fluorescence imaging⁹¹⁻⁹⁴, which are able to render both morphology profile of the sample and protein specificity. The multimodality system, H⁺iSPIM, developed in this thesis [Chapter 8] is also in line with this strategy. In addition, Chung *et al.* reported a simple hybrid system⁹⁵ of wide Field of view (FOV) fluorescence microscope with brightfield microscope using computational optics: Fourier ptychography⁶¹. The phase image retrieved from Fourier ptychography offers an estimated aberration map across the wide FOV that accurately describes the aberrations of the imaging system. This, in turn, improves the fluorescence images through the numerical image deconvolution. This study demonstrated the direct advantage of a multimodality imaging system, where the phase information corrects aberrations in fluorescence intensity images.

In summary, multimodality configurations can not only aim at providing additional images to assist the biological investigations but also can improve the image quality by post processing methods.

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Chapter 3 Dynamic morphology of anucleate blood cells

3.1 Overview of cell mechanics

The mechanical properties of cells refer to how cells interpret and respond to certain mechanical stimulus, which is increasingly recognized to play a significant role in numerous biological functions¹⁻². Mechanical phenotyping techniques³⁻⁴ such as biological atomic force microscopy, optical manipulation, and real-time deformability cytometry have become important tools for cellular mechanical studies. This chapter first briefly covers the significance of cell mechanical studies. Next, a short summary of quantification methods for applying physical forces and measuring the response of cells is provided.

3.1.1 Significance of cell mechanical study

Genetic sequencing techniques have revealed the sequences of entire nucleic acid strands of many organisms, which makes it fairly straightforward to discover protein contents within a certain cell type⁵⁻⁶. However, the physical mechanisms of how these expressed proteins are controlled and arranged to execute essential cell functions, such as migrating, deforming, and proliferating, is still not completely clear. Cell biomechanical study is aimed to solve this question. It can be defined as the study of spatial and temporal responses of cells to applied physical forces⁷⁻⁸. Currently, the field of cell biomechanics strives to combine computational⁹⁻¹¹ and experimental methodology¹²⁻¹³ to offer a precise description of cell behaviours and hence providing new perspectives for mechanics in physiological functions and disease biology. Recent advancements have included researches of the extracellular matrix (ECM) interactions, subcellular structures connectivity, injury or contraction of cells due to mechanical forces¹⁴⁻¹⁷. Even though new progress has accelerated the development of tissue engineering, three-dimensional (3D) culture, biomedical modelling, and biomaterials, our understanding of living cell biology is still in its infancy. To decipher the fundamental mechanisms of it, more high-throughput quantitative tools are needed to study cell deformations, structural dynamics, and mechanochemical studies.

3.1.2 Techniques for measuring cell dynamics

Measuring spontaneous dynamics of cells is one part of investigating the cell mechanics. The most representative methodology is Particle Tracking Microrheology (PTM). PTM technique was originally used in determining the mechanical properties of viscoelastic materials ¹⁸⁻²⁰. It depends on precise measurements of the particles trajectory, which provides parameters such as mean square displacement or autocorrelation for mechanical response function by means of equilibrium thermodynamic assumptions ²¹. Adapted for cell mechanical studies, this technique is widely used in investigating the mobility of the cytoplasm along with the spontaneous motion of cytomembrane. In PTM, micron or submicron beads are delivered into live cells and the spontaneous motions of them are recorded in a resolution of submicron and millisecond ²². The rheological properties of a cytoplasm can be calculated from the motions. Another method is called Traction Force Microscopy (TFM), which uses a polyacrylamide substrate with fluorescent microspheres embedded for detection of substrate deformation as a result of forces exerted by cell migration in two dimensions ²³ or three dimensions ²⁴. Similarly, a flexural microneedles-array substrate was another methodology, in which tips of the microneedles were used to reveal the traction forces generated by the cells cultured on the substrate ²⁵.

The most frequently performed cell mechanical investigations involve applying additional physical forces. The essential process includes generating the physical forces/loads at a single cell level and quantitatively measuring responses, such as movement, deformation and molecule signals. There are mainly three types of experimental technique to probe cells, which are mainly depicted in figure 3.1. The first technique stimulates a cell locally, for example, using atomic force microscopy (AFM) [Figure 3.1(a)] and magnetic twisting cytometry (MTC) [Figure 3.1(b)]. In AFM, a sharp metal tip (nanoneedle) of a flexible cantilever generates a local force to deform the cell surface and a feedback loop is used to adjust bending of the cantilever to maintain the applied force. The precise movement of the cantilever is measured by laser light detected through the photodiodes, which tells exactly the deformation of the cell ²⁶. This indentation later is used to estimate the elasticity and rheology of the cell ²⁷. In investigations using MTC, one or multiple magnetic beads with functionalized surfaces are

attached to a cell membrane or embedded inside the cell. The position and the movement of the beads can be controlled by an applied magnetic field ²⁸. As a result, the deformation of the cell introduced by the movement of the beads can be observed by optical microscopes. Using physical apparatus, one would need to use precise micromanipulation tools i.e. micropipette aspiration (MA) [Fig 3.1(c)] to controllably deform cells. The deformation of the cell can also be performed remotely using laser beams. The forces involved in optical manipulation with optical gradient forces ²⁹ (optical trapping, optical tweezers) in focused laser beam ³⁰. Optical scattering forces in optical stretching using two counter propagating non-focused laser beams to stretch a cell ³¹. Figure 3.1(d) shows an example of using optical trapping to manipulate a red blood cell by moving the beads attached to it. MA deforms a cell by applying suction force on the surface of a cell. The mechanical properties of biological cell calculated by mapping geometry changes in shape ³². Using microfluidic capturing wells to integrate large numbers of single MA-like structures, it is now possible to perform multiple measurements in a single microfluidic chip ³³. This improvement have led to MA march toward to a method capable of evaluating a population of cells. For example, shear-flow method generates the shear stress over the entire cells by usually a cone-and-plate viscometer ³⁴ or a parallel-plate flow chamber where cells experience a laminar flow ³⁵. Another option for multiple cells investigation is the implementation of the structured soft substrate like microneedles-array substrates introduced previously ³⁶. Using these platforms, the effects of mechanical loading, chemical or thermal stimulation, and injury responses, can be examined. In the next section, we shall cover mechanics that is specific to red blood cells (RBCs) and platelets.

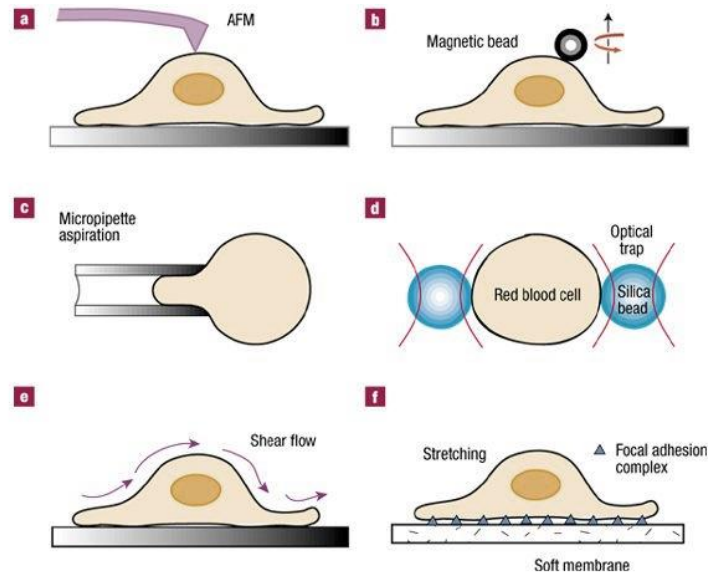


Figure 3.1 Summary of basic experimental techniques used to probe living cells, reprinted with permission from Bao et al. ¹ © 2003, Springer Nature. (a) Atomic force microscopy (AFM); (b) Magnetic twisting cytometry (MTC); (c) Micropipette aspiration (MA); (d) Optical trapping; (e) Shear flow; (f) substrate stretching.

3.2 Red blood cells

This section mainly covers the basic mechanics of RBCs. It starts with a brief overview of the structure and functions of RBCs, followed by a discussion on the impact of deformability of RBCs and how measurements of deformability relate to specific blood borne diseases.

3.2.1 Function and structure

Red blood cell (RBC), also named erythrocyte, is the largest population of cells in most vertebrates. There are roughly 20 to 30 trillion RBCs in an adult human at any time ³⁷. RBCs are derived from hemopoietic stem cells in the bone marrow. The mature RBC in the circulatory system is in an oval biconcave-disk shape. It contains no nucleus or organelles, only the haemoglobin meshwork with a lipid bilayer membrane as the sack ³⁸. Therefore, the main structure of RBCs includes three components: the fluid cytoplasm (haemoglobin), the lipid membrane, and the membrane skeleton. Haemoglobin is an iron-containing metalloprotein in charge of carrying oxygen, of which the heme prosthetic group presents the red colour. The lipid membrane of RBCs consists of phospholipid bilayer and intramembrane proteins. The membrane skeleton is formed by a

spectrin–actin protein network, which is attached to the transmembrane proteins and adjacent to the lipid membrane. The loosely packed cytoplasm, without nucleus or organelles, allows RBCs to go through the narrowest capillary with high flexibility of distortion. They are circulated within the blood vessels of different diameters (about 3 μm to 25 mm) under different wall shear stress (1-60 dyne/cm²). Gas exchange is the primary function of RBCs circulating through the whole body, during which the oxygen diffuses from the oxygenated RBCs to surrounding cells and carbon dioxide produced by cellular respiration diffuses from the interstitial fluid into the RBCs.

3.2.2 Deformability of red blood cells

The ability to greatly deform is one of the most important properties of RBCs, that not only allows RBCs to pass through capillaries but also aids them maintain unscathed when experiencing significant stress during circulation ³⁹. Hence, the decrease in deformability can cause detrimental repercussion immediately. Deformability of RBCs is typically described in terms of viscosity, shear modulus, and bending modulus, which comes from two contributions. The first part is the rheological properties of the intracellular fluid of RBCs, which is ruled by hemoglobin concentration and physicochemical properties of it. The second contributor is the rheological properties of RBC membrane and membrane skeleton. Any disturbance in the internal and external environment can trigger the RBCs deformability changes, particularly in many pathological conditions ⁴⁰. For example, the variations in osmotic conditions ⁴¹, the increase in intracellular calcium ion ⁴², temperature variation (hypothermia) and the alteration in membrane composition ⁴³ will all affect the deformability of RBCs ⁴⁴.

RBC abnormalities in hematological disorders and diseases are often accompanied by altered RBC deformability. For example, sickle cell anemia ⁴⁵ is caused by the genetic defect of molecular structure of hemoglobin S (HbS) ⁴⁶ which undergoes gelation and fiber formation during deoxygenated process, which changes intracellular fluid to a viscoelastic material and decreases the deformability ⁴⁷. Another example of decreased deformability is malaria infection ⁴⁸. Malaria parasites which enter RBCs cytoplasm not only change the internal space by introducing the parasite itself but also modify the cell membrane by

expressing the intramembrane proteins. Both alterations reduce the deformability of the RBCs dramatically during the parasite life cycle. The detailed pathology is discussed in chapter 6.1.

3.2.3 Measuring deformability of red blood cells

The mechanical properties of RBCs have been widely studied since last century ^{35, 39, 49}. One important aim of these RBCs biomechanics studies is to provide further insights to fill in gaps of current pathophysiology knowledge and lead to new methods of early diagnosis and effective treatment. Initial biomechanical approaches to studying RBCs rheology mainly includes biochemical methods, simple shear flow approaches (cone-plate viscometer and counter-rotating rheoscope), MA and optical tweezers ⁵⁰⁻⁵¹. During the exploration, chemical stiffening methods ⁵² have been established for manipulating the RBCs deformation. For example, diamide ⁵³, a sulfhydryl reagent which oxidizes sulfhydryl groups to the disulfide form, primarily affects the membrane stiffness of RBCs and affects no strain rate of cytoplasm. Glutaraldehyde ⁵⁴⁻⁵⁵, as another frequently used chemical, causes cross-linking of membrane proteins as well as haemoglobin, which increases the effective viscosity of both lipid membrane and cytoplasm, and in turn reduces the deformability of entire RBCs. These chemical approaches helped in establishing the knowledge of comprehensive RBCs rheology and developing the practical models ⁵⁶⁻⁵⁸ for simulation studies.

In 1971, Miller et al. ⁵⁹⁻⁶⁰ studied malarial parasites infected mammalian RBCs by viscometer and cell-filtration methods, which was the first study indicating that the deformability of RBCs could be impaired by infection of malarial parasites. A comprehensive investigation of mechanical properties of healthy and malarial parasite infected RBCs at different stages was conducted using optical tweezers in 2004 by Mills et al. ⁶¹. Around the same time, a microfluidic device was first used to investigate the blockage of infected RBCs passing through the different sizes of narrow elastomeric channels ⁶². Similar to malaria studies, the mainstream of sickle RBCs mechanical study used optical tweezers ⁶³ and MA ⁶⁴ in the past. With the development of nanostructure and microstructure fabrication, microfluidics technique has been increasingly applied in sickle RBCs mechanics investigation. It provides the platform for manipulating

a large population of RBCs and the opportunity of observing individual rheological behaviours.

3.3 From platelets to thrombus

Platelets, another specialized anucleate blood component, are essential for maintaining hemostasis, and preventing excessive blood loss from an injured blood vessel. The process is one of the primary protective mechanisms in our bodies, and is one of the biological subjects in the thesis (chapter 7). Significant morbidity and mortality occur when the process is out of balance. In this section, the background of how platelets aggregate and form a thrombus is introduced and a brief interpretation of different pathological disease and related research investigation are provided.

3.3.1 Platelet structure and function

Platelets, also called thrombocytes, circulate as smooth-surfaced discoid cellular fragments⁶⁵⁻⁶⁷ with a diameter of 2-3 μm and thickness of 0.5 μm in a resting state. The normal platelet count is around 150,000-350,000 per microliter of blood in a healthy human body⁶⁸. The cytoplasm of the platelet possesses all kinds of organelles found in other mammalian cells except for a nucleus⁶⁵. The membrane of the platelet contains a wide variety of mobile receptors that play a prominent role in platelet hemostatic function by interacting with and responding to vascular adhesive proteins and soluble platelet agonists. Figure 3.2 shows the major platelet receptor-ligand interactions (adapted from Storey, 2008⁶⁹), including integrins ($\alpha_2\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_v\beta_3$)⁷⁰⁻⁷¹, leucine-rich repeated receptors (Glycoprotein [GP])⁷², immunoglobulin superfamily proteins (GPVI⁷³, Fc γ RIIA), G-protein coupled seven transmembrane receptors (PAR-1 and PAR-4 thrombin receptors, P2Y₁ and P2Y₁₂ ADP receptors, TP α and TP β TxA₂ receptors)⁷⁴⁻⁷⁵. The surface receptors shown in the figure represent the key signalling pathways that allow platelets to respond to individual stimuli specifically and lead to activation, morphological alteration as they form a clot to stop excessive bleeding. Platelets have also shown to influence immune responses by means of these signalling pathways, which is fully discussed in reviews⁷⁶⁻⁷⁸ and is not discussed here.

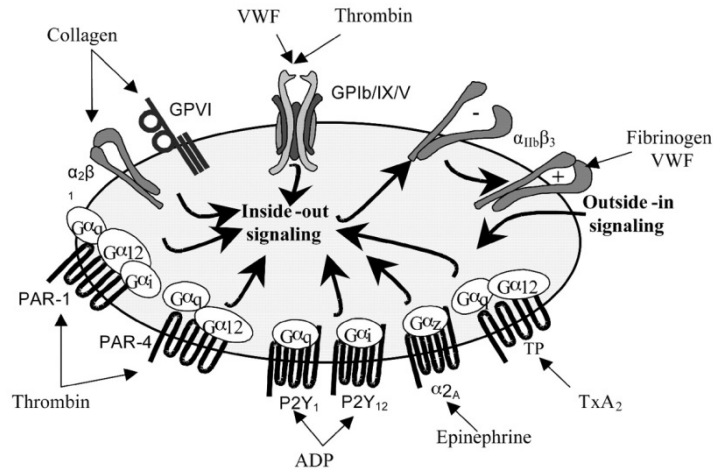


Figure 3.2 Major platelet receptor-ligand interactions, adapted with permission from Storey, 2008 ⁶⁹. The surface receptors represent the key signalling pathways that allow platelets respond to individual stimuli specifically.

3.3.2 Platelet activation, aggregation and thrombus formation

Hemostasis ⁷⁹⁻⁸⁰ serves to maintain the integrity of the circulatory system. The process is synergistically regulated by two main factors ⁸¹: soluble platelet agonists and blood flow strain ⁸²⁻⁸⁴. The former associates with plasma components, platelet membrane receptors, intracellular signalling pathways ⁸⁵ and the latter is related to the local shear conditions. A cascade of events towards to hemostasis can be briefly broken into three steps; (1) The initial activation and adhesion of platelets to the damaged vessel wall, (2) Platelet aggregation, (3) Thrombus formation and stabilisation (fibrin generation).

During the process, the circulating platelets contact the damaged vessel wall, in which collagen is exposed. A blood glycoprotein, the Von Willebrand factor ⁸⁶ that is present both in plasma and inside platelets, as well as in endothelial cells and the sub-endothelial matrix of the vessel wall, captures platelets to the collagen fibre by attaching to a specific receptor on the platelet membrane. Then the attached platelets become activated and release secondary agonists/mediators to recruit more platelets to the adhesive platelet mass ⁸⁷⁻⁸⁸. More and more platelets are recruited to the growing platelet mass by developing contacts between platelets, cumulatively building a thrombus. Soluble fibrinogen is converted into insoluble strands of fibrin around the platelet plug by

serine protease thrombin, which promotes the stabilization of the plug to provide continued protection from bleeding until the damaged vessel is repaired. During this final step, the RBCs and white blood cells are also recruited to the plug to form the final product, thrombus.

3.3.3 Diseases and imaging methods

Under pathophysiological conditions, any perturbation or disorder can lead thrombus formation process, supposed to be a normally protective cascade sequence, becoming insufficient or excessive. The result could either be haemophilia, Von Willebrand disease (VWD) ⁸⁹ or arterial thrombosis, deep vein thrombosis ⁹⁰⁻⁹¹. Either way is a fatal clinical event. Another fatal disease can be caused by thrombus, or more specifically the embolus of thrombus, is the ischaemic stroke ⁹². When a thrombus or a fragment of thrombus gets detached from the adherent site to the circulation, it could be lodged at the narrow part of the vessel and eventually interrupts the blood supply to the brain, which is extremely dangerous (e.g. stroke). Due to the complexity of thrombus formation and dynamics (multi-factors dominated event), the current understanding of the related mechanism is still limited by the tools available to image them *in situ*. Over the last decades, the development of fluorescence imaging technique has led to the discovery of major receptor-ligand interactions. Current studies of platelets are generally focused on a deeper understanding of the finely regulated molecular mechanism. The investigation methods can be classified into two main regimes, individual platelet interactions and platelet aggregates (thrombus). These regimes are investigated using a range of imaging and computational methods. Figure 3.3 demonstrates a summary of the investigations by different imaging techniques and observing perspectives. At the single platelet level, Poulter *et al.* ⁹³ utilized scanning electron microscopy (SEM) to scrutinize the structures of platelets with high definition [Figure 3.3 a) i)], also applied structured illumination microscopy (SIM), direct stochastic optical reconstruction microscopy (dSTORM) ⁹⁴, as well as live-cell total internal reflection fluorescence microscope (TIRF) microscopy ⁹⁵ to characterize signalling pathways [Figure 3.3 a) ii)]. The observation was aimed to gain a better understanding of actin nodule organization and function, which is a novel F-actin structure present in platelets during early

spreading. Novel patterned hydrogel-based microfluidic device [Figure 3.3 a) v)], was used for investigating the single-platelet nanomechanics ⁹⁶. Single-platelet force spectroscopy [Figure 3.3 a) iv)], is used to rupture forces between two platelets ⁹⁷. For platelets to thrombus, Laser scanning microscopy, discussed in chapter 2, was widely used to investigate the coagulation process *in vivo* [Figure 3.3 b) i)] using animals. Well-designed microfluidic chips [Figure 3.3 b) iii)] were applied to *in vitro* thrombosis study in a more controllable way ⁹⁸. Quantitative phase microscopy (QPM) also has been successfully used to quantify individual adherent platelet ⁹⁹ [Figure 3.3 a) iii)] or individual thrombus ¹⁰⁰⁻¹⁰¹ *in vitro* without staining [FIG.3.3 b) ii)]. In the clinic, Optical Coherence Tomography (OCT) and ultrasound are typically used to detect the thrombus in the vessel for inspection and diagnosis ¹⁰²⁻¹⁰³. Since flow condition is another key feature for thrombus formation and embolus. The computational modelling ¹⁰⁴ has emerged to simulate the flow dynamic in atherosclerotic plaques, which was considered to be promising to tackle the shear-dependent mechanisms of thrombus formation ⁸²⁻⁸³. An example was shown in figure 3.3 c).

Techniques mentioned above covers most of the platelet function investigation methods. In this thesis, QPM technique was chosen to observe the thrombus dynamic due to its ability of imaging non-stained sample and video-rate capturing with digital holographic microscopy system (chapter 5).

formation by QPM, reprinted from Ref. ¹⁰¹ © 2013 Optical Society of America. iii) Microfluidic device with controllable wall shear to form the thrombus, reprinted from Ref. ⁹⁸ with permission from The Royal Society of Chemistry. c) Computational model for studying endothelium around atherosclerotic plaques, reprinted from Ref. ¹⁰⁴ licensed under a Creative Commons Attribution 4.0 International License.

3.4 References

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Chapter 4 Microfluidic tools for cell biological studies

Microfluidics is a technology that manipulates small volumes of fluids or small samples in fluids. Microfluidic devices are greatly useful in a lot of fields by offering a better and more compact presentation of complex systems [1]. In this thesis, microfluidic tool serves to replicate physiological flow in biological experiments in chapter 6 and chapter 7. This chapter mainly focuses on the microfluidics in cell biological and clinical applications. The chapter starts with the physical principles of the fluid, rendering the basis of the microfluidic technique. Then it introduces the common fabrication technique, soft lithography fabrication process adopted in the works [Chapter 6, 7] of this thesis. In the end, the chapter finishes with a review of current microfluidic applications in biological cell studies.

4.1 The physics in microfluidics

Fluid flow at the microscale or nanoscale turns out to have unique features that can be beneficial to manipulation and assistance for biological studies. The physics of fluid at microscale and nanoscale are the foundation of design and principle of microfluidic devices. This section introduces the physics in microfluidics concisely.

4.1.1 Viscosity, velocity and shear stress

Two inherent properties of a fluid, density ρ and viscosity μ , govern the behaviour of it. Viscosity describes the resistance of a fluid to a gradual deformation induced by shear or tensile stress. For most of fluids used in microfluidics for cell studies, saline solution for example, the viscosities of them are constant, which are also called Newtonian fluids. These fluids obey Newton's law of viscosity [Figure 4.1] which states that the shear stress τ between adjacent fluid layers is proportional to the velocity gradient between the two layers. The kinematic viscosity, the ratio of fluid viscosity to density, is also used to characterise fluids in some circumstances.

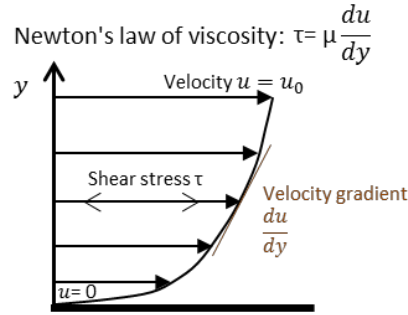


Figure 4.1 Diagram of Newton's law of viscosity. Dynamic viscosity μ , shear stress τ , velocity u , distance y .

4.1.2 Laminar and turbulent flow

One of the most cited parameters that describe flow regime is a dimensionless parameter, the Reynolds number (Re) ¹⁻². This parameter is given by the ratio of inertial forces over viscous forces in the flow. In the microfluidic channel, the Re can be calculated by

$$Re = \frac{\rho u D_h}{\mu}$$

where ρ is fluid density, u represents characteristic velocity and μ is the viscosity of the fluid. D_h is the hydraulic diameter which is a parameter calculated by the channel's geometry dimensions. Re also can be seen as a tendency of a fluid to become turbulent. Therefore, low Reynolds numbers ($Re < 2300$) generally indicates that viscous forces dominate the flow regulation. The flow is defined as laminar flow since it flows in parallel layers perpendicular to the direction of the flow and there is no disruption between the layers. While high Reynold numbers ($Re > 2300$) indicates that the flow becomes dominated by inertial forces and the fluid motion is chaotic. The figure 4.2 is a simple schematic diagram of turbulent flow and laminar flow.

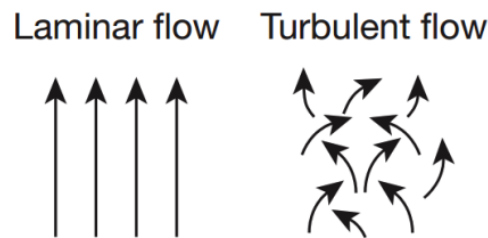


Figure 4.2 Schematic diagram of laminar flow and turbulent flow.

Laminar flow is primarily used in microfluidics, where the direction and the rate of the velocity of small particles (cells for example) in the fluid stream are known and controllable. Because of the micron size of channels in microfluidic devices, the laminar flow is easily formed and serviceable to deliver the particles or cells. While turbulent flow can be obtained by designing obstacle-like structures in the microfluidic device for certain applications ³.

4.1.3 Surface tension, hydrophobic and hydrophilic effects

Surface tension force at microscale is significant ⁴, which is induced by the cohesive forces between liquid molecules. At the surface of the liquid, the liquid molecules are in contact with the outside medium (usually gas molecules). The cohesive forces between these outermost liquid molecules are stronger than those inside the liquid, which are all surrounded by same liquid molecules. This stronger cohesive force bends the surface of the liquid to reach shorter molecules linkage. Similar phenomena of liquid on a surface are hydrophobic and hydrophilic effects. Hydrophile describes a material or molecule that is attracted to water molecules while hydrophobe means the material or molecule repelled to water molecules. The attractive or repulsive force makes the material contact with water droplet with a certain angle, which is a balanced result of intermolecular forces.

In a microfluidic device, surface tension is equally as important as friction or drag force. In a nanofluidic device, the surface tension force becomes almost dominant owing to scale effect. Consequently implementation of surface tension force would be effective in micro/nano fluidic systems, for example surface tension driven fluidic systems ⁵⁻⁷ have been developed and utilized in various fields. The hydrophobic and hydrophilic effects are also exploited in microfluidic devices for a variety of purposes. The surface modification is sometimes required to reach certain hydrophobic or hydrophilic result. For example, certain surface hydrophobicity gradient is needed for droplet manipulation applications ⁸⁻¹⁰. Polydimethylsiloxane (PDMS), a commonly used material for bio-applications, is naturally hydrophobic. It causes microchannels tend to trap bubbles and suffer nonspecific adsorption to cells and proteins ¹¹. Therefore, surface modification is usually required for PDMS chips ¹²⁻¹³.

4.2 Fabrication methods

In this section, different fabrication methods for microfluidic device are introduced. The emphasis of the section lies on the soft lithography which is the method adapted in most of the work [Chapter 6, 7] in the thesis. The 3D printing technique is also used in microfluidic devices and covered in this section with other methods.

4.2.1 Soft lithography

Soft lithography technique ¹⁴ is a fast and cost-effective fabrication method widespread in microfluidic tools for cell biological research. To explain the soft lithography process in simple terms, an elastomeric material (polymers) ¹⁵ is utilized to replicate the microstructures on a positive master mold and later the sculpted elastomeric material is attached to a substrate, usually a glass slide, to form a microfluidic chip. The technique allows rapid prototyping of microfluidic devices for testing new concepts developed by biologists. The dominant material used in soft lithography is poly dimethylsiloxane (PDMS) ¹⁶ which is a nontoxic, optically transparent elastomer with outstanding thermal and chemical stability as well as insulating property. The detailed physical and chemical properties of PDMS were reviewed in Ref. ¹⁶.

In the first step of fabricating PDMS microfluidic devices, a master mold with desired channel structures is manufactured usually by photolithography technique. Micromachining ¹⁷ can also be used to manufacture the mold for higher precision. In the work presented in this thesis (chapter 5 and 6), photolithography technique was adapted for effective and low-cost fabrication. Figure 4.3.a) demonstrates the process of it. A silicon wafer is used as the substrate. The preparation of the wafer is removing moisture by heating it at 120°C for 15 min, which is beneficial for photoresist stick on it. Then a thin layer of photoresist is coated on surface of the wafer by Spin coating method. The thickness of the photoresist layer was controlled by programmed spinning speeds. Baking the photoresist is required for evaporating the solvent and making the photoresist more solid. Next the wafer with photoresist was placed under UV exposure with a cover of photomask. The photomask is an opaque film or glass with transparent microchannel-pattern areas where the UV light can pass and activate the curing

reaction of the photoresist. Then the second photoresist baking is performed to provide the energy for continuing curing. The final step is photoresist development, where the non-linked photoresist is diluted in the developer. The developer is a solvent mainly composed of Propylene glycol monomethyl ether acetate (PGMEA). After that the positive master mold is silanized by TriMethylChloroSilane (TMCS) treatment, in order to gain a hydrophobic surface. Otherwise, the surface would be too hydrophilic for PDMS to be peeled off. After this, the mold is ready for PDMS replication and is quite robust for large number of repetitive replications. More specific procedures of mold photolithography preformed for fabricating the microfluidic devices used in later chapters are provided in Appendix A1. Figure 4.3 b) shows the process of soft lithography. It simply can be divided into two steps: PDMS replication and microfluidic chip making. PDMS is prepared with mixing curing agent and PDMS monomers in a 1:10 (w/w) ratio. For specific applications, the ratio can be adjusted to have a softer or harder PDMS. The mixture is then degassed in a desiccator to get rid of air bubbles. The obtained clear PDMS is poured onto the mold in a petri dish and cured in an oven for 2 hours at 80°C. Once cooling down, the PDMS can be peeled off from the mold and be cut into individual chips. Then the inlets and outlets area of the microchannel are pierced for tubing connection. Finally, to bond the PDMS to the glass slide, surface of PDMS (the microchannel side) and glass slide are plasma treated in order to obtain hydroxy group (Si-OH). Then the PDMS is simply pressed on a glass slide, with the functionalized side contacting the glass, leading to a strong and permanent bond (Si-O-Si).

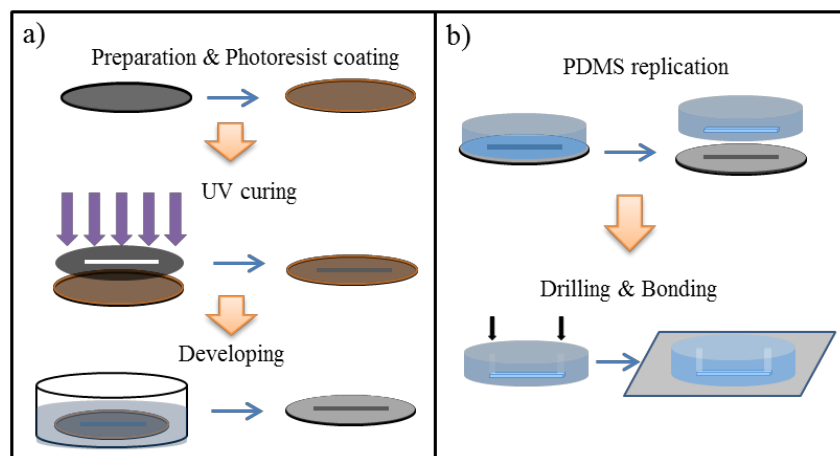


Figure 4.3 a) Mold photolithography process. b) PDMS soft lithography process. Detailed process can be referred to Ref.[14].

4.2.2 Other methods

There is a variety of other fabrication methods which include micromolding¹⁸, laser ablation¹⁹⁻²⁰ and micromachining²¹. Those techniques are more costly, labour intensive and less throughput in terms of fabrication compared to soft lithography. Apart from them, three-dimensional (3D) printing has been increasingly used in microfluidic fabrication due to its low cost, fast and flexible fabrication ability in recent years²²⁻²⁴. Various branches of 3D printing technique have been developed with slightly different approaches²⁵. Different 3D printing techniques have been reviewed in Ref.²⁶ thoroughly. They can be categorized into two main types: solid material extrusion and lamination methods, and liquid resin inkjet/lithographic methods. For example, Fused Deposition Modeling (FDM), also called Fused Filament Fabrication (FFF), is the most common 3D printing process that belongs to the solid material extrusion and lamination family. It uses a hot nozzle to melt and extrude thermoplastic filament on a plate in programmed locations continuously and build the 3D microchannel layer by layer. As one of the most simple and straightforward 3D printing methods, FDM was first demonstrated as a method for fabricating positive master mold for soft lithography in 2002²⁷. As the accessibility and the resolution of the technique has evolved, FDM printing was implemented for the direct fabrication of microfluidic chips in 2012²⁸. An example in liquid resin lithography is the stereolithography (SLA) technique, creating the 3D microchannel structures by curing a polymer layer by layer using a UV laser beam. Recently, the two-photon SLA²⁹ has been proposed and implemented. Replacing the UV laser by a laser of centre wavelength of 780 nm, two-photon excitation can be achieved to cure the polymer more precisely and faster. In all, the 3D printing technique is in rapid growth. More and more complicated and fine microchannel structures have been achieved, 3D micromixers³⁰ and integrated valves³¹ for example. The prospect of the technique in microfluidic fabrication is promising.

4.3 Microfluidic in cell studies

In the field of cell studies, the focus is always on both census of large population and measurements of individual behaviours. Therefore, the discussion is divided into two subsections. The first part is about high throughput microfluidic devices for cell study while another subsection focuses on novel microfluidic devices for individual cell mechanic study.

4.3.1 High throughput microfluidic devices for cell sorting and disease diagnosis

A key advantage of microfluidic devices is the ability to performing high throughput tests in a low-cost and miniature system. This compelling advantage is in high demand in the field of biological cell studies. The development of high-throughput microfluidic flow cytometry³²⁻³³ is a classic example, which provides multi-parameter measurement of bulk cell samples. The applications of it covers new drug development³⁴⁻³⁶, disease diagnosis³⁷⁻³⁸, gene engineering³⁹⁻⁴¹ and so on. One of the most fruitful applications can be disease diagnosis⁴²⁻⁴⁴, especially the in haematology⁴⁵ and immunology, where blood or plasma is the main object. The simple, low cost, on-site diagnosis conferred by microfluidic chip is greatly helpful for point of care service and leads to higher rates of treatment in developing countries. Figure 4.4 shows a microfluidic chip for enzyme-linked immunosorbent assay for diagnosis of HIV (human immunodeficiency virus), developed by Chin *et al.*⁴⁶. It generates the outcome within 20 min and only need a few microliters of blood sample. Importantly, the assay requires no external pumping systems. A disposable syringe can be used to generate the vacuum for fluid actuation.

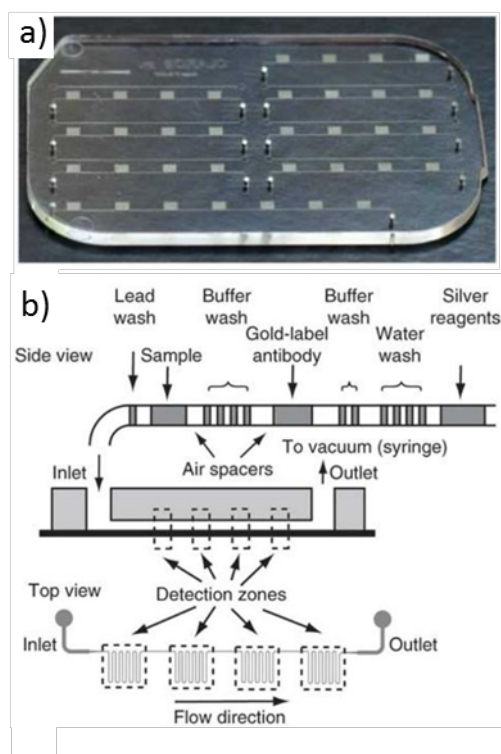


Figure 4.4 An enzyme-linked immunosorbent assay for diagnosis of HIV, reproduced from Ref.⁴⁶ with permission from © 2011, Springer Nature. a) Photo of the microfluidic chip. b) Schematic diagram with a side view and a top view of the assay. It offers a passive delivery of multiple reagents. A disposable syringe is used to generate the vacuum for fluid actuation.

Another rapidly growing application of high throughput microfluidic devices for cell studies is a variety of designs for cell sorting and cell separation⁴⁷⁻⁵². Both external forces (e.g., acoustic, magnetic, electric, and optical) and inertial forces (fluidic shear, adhesion mechanism, cell deformability and cell motility for example) are utilized to separate the cells or purify the cell populations. Microfluidic cell sorting technique provides myriad advantages over the conventional methods by minimize the potentially biohazard, reducing the size of equipment, and simplifying the operation, which make it available for even non- technical users. A review of current microfluidic cell sorting methods has been provided by Ref.⁵³.

In all, by rendering the ability to work with smaller reagent volumes, shorter reaction times, and parallel operation, microfluidic technique has the potential to change the manner how modern biological study is performed.

4.3.2 Microfluidic devices for cell deformability

Microfluidic device has unique advantages for cell mechanism studies. Since the fluidic environment in a confined microchannel or micron chamber is safe and beneficial for cell culture⁵⁴⁻⁵⁵ and also is convenient for cell manipulation⁵⁶. The shear stress induced by simple or complicated microstructures, additional applied forces by electric or optical field, and the chemical stimulus can all be employed to induce cell dynamics. With the cooperation with advanced imaging techniques, plenty of cell mechanical studies have been performed using microfluidic methods over the last decade^{55, 57-60}. Ref.⁶¹ provided a summary of the applications. Cell deformability measurement is one of the most frequently used methods that has been applied over various cell types⁶²⁻⁶⁴. Morbid RBCs and cancerous cells are two of the most popular objects that draw attention mostly, since the investigation of the deformability of them brings out better understanding of the related pathological mechanism. Two common methods to deform the cells are funnel constrictions and hydrodynamic stretching which is demonstrated by two examples in Figure 4.5. The geometrical parameters of the cell shape were used to represent the deformability in both cases. Most of the investigation used inverted fluorescent microscopy or phase contrast microscopy to capture the videos of the stretched cells. As a result, only two-dimensional deformation is characterised. It would be unrivalled that the three-dimensional shape of the cell could be captured to quantify the deformation for better and more comprehensive analysis of cell mechanic. This aim is achieved in the thesis and presented in chapter 6.

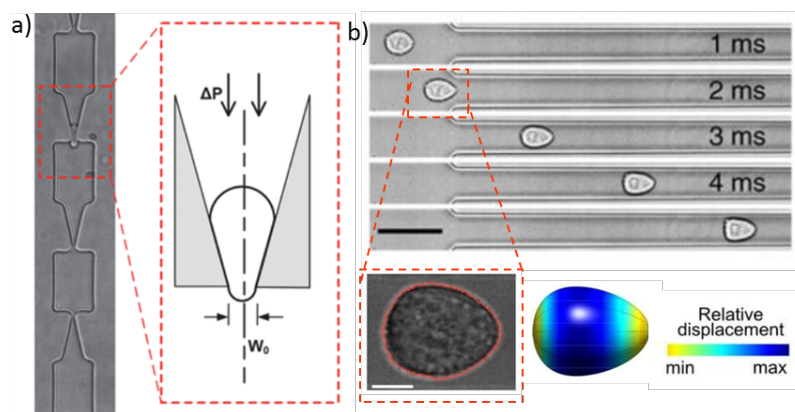


Figure 4.5 a) Cell deformation induced by funnel constrictions, reproduced from Ref.⁶⁵ with permission from The Royal Society of Chemistry. b) cell

4.4 References

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Chapter 5 Automated Off-axis digital holographic microscopy system

Publication relevant to this chapter:

He X, Nguyen CV, Pratap M, Zheng Y, Wang Y, Nisbet DR, Williams RJ, Rug M, Maier AG, Lee WM. “Automated Fourier space region-recognition filtering for off-axis digital holographic microscopy” *Biomedical optics express*. 2016 Aug 1;7(8):3111-23.

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Contribution of others:

The work related to the cell segmentation processing was done with the guide of Nguyen CV. The work related to the user graphic interface was done with the team work between Zheng Y and me. RBCs sample was provided by Pratap M. Murine primary cortical neurons, neural stem cells, and the hydrogel samples were provided by Wang Y.

5.1 Background

Traditional light microscopy relies on variations of light intensities reflected from or transmitted through the sample. Thus, cells or thin tissues with high transparency result in only slight intensity changes of the light. With any photodetector, this low intensity change gives extremely low contrast images. However, the phase changes of light that are induced by the sample are abundant but cannot be directly detected. This reality has triggered researchers to explore how to implement the interference and diffraction in a microscope to reveal the phase information of the samples. Optical microscopy that quantitatively retrieves both phase and intensity of the light is named quantitative phase microscopy (QPM). The basic theory of them can date back to Thomas Young's 1801 discovery of double-slit interference¹. In this chapter, one of the QPM techniques, digital holographic microscopy, is adapted. Also, the automated operation and imaging process was built for the proposed microscopy system.

5.1.1 Theory of holography

The essential principle of digital holographic microscopy (DHM) is the measurement of microscopic phase shift through classical interferometry and modern Fourier optical transforms. The small transparent structures of the object do not introduce obvious intensity change but do introduce the phase change of light. The phase information can be encoded through an interferometry

configuration, which is intensity information and can be recorded easily. These phase changes from the sample can be displayed as disturbances on the interference fringes. The equations (1) and (2) represent the reference wave going through unobstructed and object wave going through the sample. R_0 and O_0 indicate the amplitude of reference wave and the object wave. The ω_1 indicates the angular frequency of both waves. The $\vec{k} * \vec{r}$ indicates the dot product of wave vector and optical length. The φ in equation (2) is the phase changes produced by the sample.

$$R(\vec{r}, t) = R_0 \exp[j(-\omega_1 t + (\vec{k} * \vec{r}))] \quad (1)$$

$$O(\vec{r}, t) = O_0 \exp[j(-\omega_1 t + (\vec{k} * \vec{r})) + \varphi] \quad (2)$$

According to wave equation, the hologram pattern (I_H) in original in-line setup can be written below:

$$I_H = |O + R|^2 = (I_o + I_r) + OR^* + RO^* \quad (3)$$

Combined with wave equations of reference wave (1) and object wave (2), the hologram pattern can be rewritten in equation (4):

$$I_H = O_0^2 + R_0^2 + |O_0 R_0| e^{j(\varphi)} + |R_0 O_0| e^{j(-\varphi)} \quad (4)$$

As shown from above equations, the phase change φ produced by the sample can be located at both $O_0 R_0^* e^{j(\varphi)}$ and $R_0 O_0^* e^{j(-\varphi)}$. Those two phases are inverted and overlap in the frequency domain. These are called twin images in an in-line arrangement. The overlapping twin images cause blurring and reduce the image quality which complicates the phase reconstruction process. However, when the two waves interference at a particular angle, the object wave O_{off} and reference wave R_{off} have a slightly different incident angle, which causes the different $\vec{k}$ in two waves. Therefore, the reference wave and object wave can be rewritten as below:

$$R_{off}(\vec{r}, t) = R_0 * \exp[j(-\omega_1 t + (\vec{k}_r * \vec{r}))] \quad (5)$$

$$O_{off}(\vec{r}, t) = O_0 * \exp[j(-\omega t + (\vec{k}_o * \vec{r})) + \varphi] \quad (6)$$

Then the hologram can be rewritten as:

$$I_{H_off} = O_0^2 + R_0^2 + O_0 R_0 * e^{j(\varphi + (\vec{k}_o - \vec{k}_r) * \vec{r})} + R_0 O_0 * e^{j(-\varphi + (\vec{k}_r - \vec{k}_o) * \vec{r})} \quad (7)$$

As shown, two factors $O_0 R_0 * e^{j(\varphi + (\vec{k}_o - \vec{k}_r) * \vec{r})}$ and $O_0 R_0 * e^{j(-\varphi + (\vec{k}_r - \vec{k}_o) * \vec{r})}$ are in symmetrical locations in spatial frequency domain, while the zero order $O_0^2 + R_0^2$ is located in the origin point (center point) of spatial frequency domain. Therefore, it's easy to separate them and retrieve the phase φ in the numerical reconstruction.

5.1.2 Retrieving phase in DHM

In digital holographic microscopy (DHM) system, numerical methods hold the key to accurate decoding of the phase information from the interference pattern or called digital hologram. The numerical steps used in off-axis DHM technique includes: discrete 2D Fourier transform, spatial frequency filtering², numerical propagation (digital refocusing if required), phase unwrapping, aberration correction³. As mentioned above, the slight tilt angle α in an off-axis arrangement can spatially separate virtual image and the true image in frequency domain, shown in figure 5.1, (fast Fourier transfer) FFT image. The mathematical description of this is equation 15. The first non-constant term describes the positive complex amplitude information whilst the second non-constant term describes the virtual imaging information. Since these two terms are complex conjugates and the base of them is mathematical constant e. After Fourier transformation, these two terms appear in two symmetrical positions in the frequency domain.

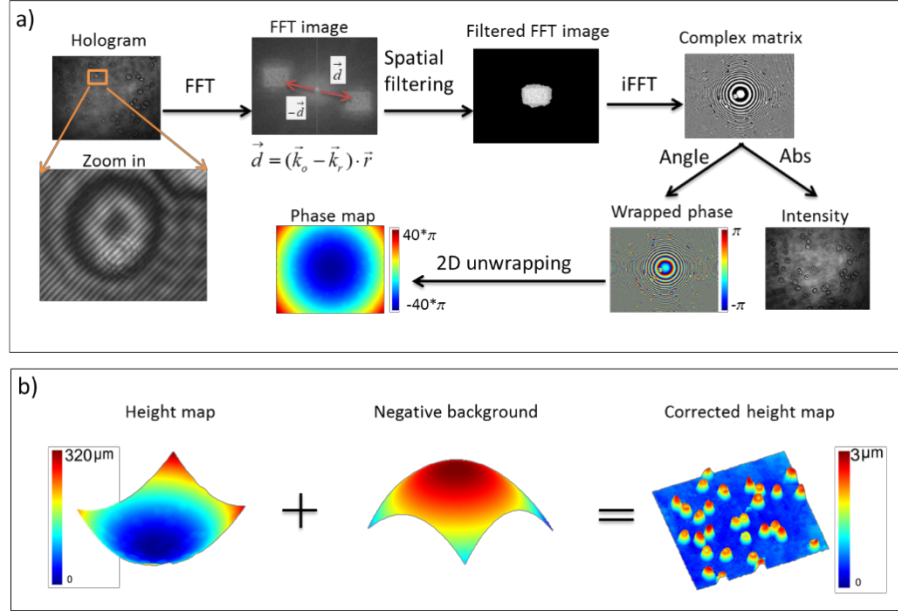


Figure 5.1 (a) Phase retrieval process; FFT, Fast Fourier transformation; iFFT, inverse Fast Fourier transformation; Angle calculation (Angle); Absolute value calculation (Abs). (b) Background removal process.

Figure 5.1 (a) shows the flow chart of the phase retrieval process. Firstly, the Fast Fourier transformation (FFT) is applied to the recorded hologram to get the frequency domain image (FFT image). Next, a digital mask is applied to the FFT image to filter out the first order and move it to the centre as the filtered FFT image. Later the inverse Fourier Transform is applied to the centred FFT image and a complex matrix is obtained. The intensity image (amplitude information) can be obtained by taking the absolute value of the complex matrix, while the phase map can be acquired by angle calculation of the complex value of the matrix. The acquired phase map is folded or wrapped within 0 to 2π . To get the continuous phase value corresponding to the optical thickness of samples, a 2D unwrapping process needs to be operated to get final unwrapped phase map. However, the phase value we get from the hologram is non-simulated data, which contains noise from the stray light and the CCD. So the simple unwrap function in Matlab is not powerful enough for the noise sensitive unwrapping processing. We implemented the 2D-SRNCP phase unwrapping method⁴ to achieve credible phase map. After unwrapping, the next operation is to calculate the optical thickness of the sample. In this process, the below equation will be needed, in which $\varphi(x,y)$ is the phase value we get from above steps, λ_0 is the wavelength of

the laser light and the Δn is the refractive index difference between the sample and the media.

$$h(x, y) = \frac{\varphi(x, y)\lambda_0}{2\pi\Delta n}$$

The height map of the sample can be plotted using the $h(x, y)$ value. The obtained height map is accompanied by a background curve if the reference wave goes through slightly different optics with the object wave. For example, there is no objective lens in the reference wave. In order to remove the background, an aberration mask (negative background in figure 5.1 (b)) is calculated from the Zernike polynomials fitting of the original height map to get the final corrected height map.

To achieve high throughput cells investigation or diseases diagnosis, these numerical calculations should be programmed to be automated and adaptive to different sample and imaging conditions. The complexity lies in the spatial frequency filtering process. Since the distribution of spatial frequencies of different samples varies significantly, the filter window needs to be adjusted correspondingly for the precise extraction of the desired frequency orders (first orders). One straightforward filtering method for precise extraction is manually selecting the first order^{3,5} in the spatial frequency domain. However, the manual spatial filtering is time-consuming and depends on subjective judgment, which is not suitable for dynamic reconstruction. One of the automatic filtering methods requires setting the threshold to obtain the spectrum and extract its boundary as the boundary of the final filtering window⁶. This raises difficulties in defining the spatial filter because of the blurry boundary and non-regular distribution of the spectrum. Obviously, this approach requires manual input for each hologram, which is therefore not optimal for automation. While there are some histogram analysis techniques⁶⁻⁷ aiming to provide adaptive filtering, unfortunately, those histogram analysis processes still require manual intervention (e.g. initial windowing parameter) to calculate the appropriate threshold. Hence, these techniques are still unable to provide sufficient flexibility or simplicity for automated selection of the appropriate spatial filter for a wide variety of samples especially confluent live cell cultures with highly scattering backgrounds.

Therefore, we developed an adaptive spatial filtering for phase reconstruction, which will be introduced thoroughly in chapter 5.3.

5.2 System design

In this section, the specific system design is demonstrated in terms of optics hardware, user interface, and the automatization algorithm.

5.2.1 Optical setup

Figure 5.2 depicts the schematic of experiment setup. HeNe laser (35 mW, model 107B-2 Spectra-Physics) is employed to provide the coherent laser light ($\lambda=633\text{nm}$). The laser light is first coupled into a single-mode optical fiber splitter and equally divided into object and reference waves. The object wave from the sample is collected by the objective lenses (Olympus 10 $\times$, NA 0.25) and projected onto the CCD camera (Retiga-2000R Fast 1394 Color Cooled) by a beam splitter (BS). Then the reference wave is superimposed on the CCD with a slight tilt angle against the object wave front. Thus, off-axis holograms are generated and recorded on the CCD plane. Also, the slightly tilted angle θ is shown in the small figure near the CCD, in which k_r and k_o indicate the incident directions of reference wave and object wave.

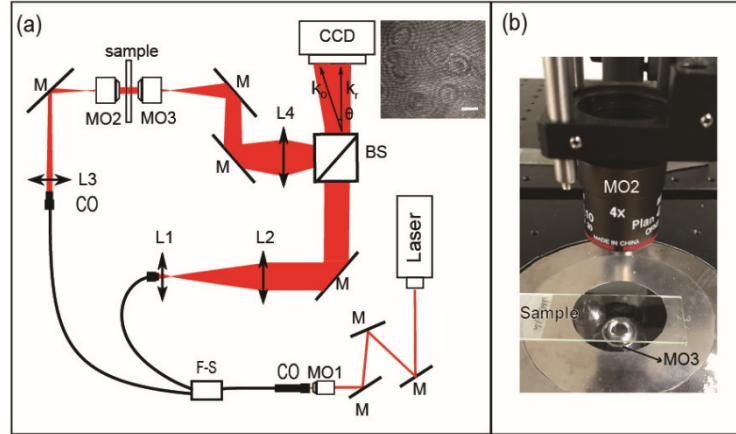


Figure 5.2 DHM Imaging setup. (a) L1 and L2 expand the reference beam. L3 focuses object beam onto the back focal plane of a second microscope objective MO3. L4 is a tube lens. M are silver-coated reflective mirrors to fold the beam along the imaging system. FS is fiber splitter and BS is the beam splitter for splitting and recombining the sample and reference beam. MO1 is the fiber coupling objective, MO2 is the illuminating objective and MO3 is the imaging objective (inset shows is an example of a recorded hologram

(scale bar = 5 μm). (b) Image showing the sample placed between the two objective lenses in the setup.

5.2.2 Graphical user interface

To make a user-friendly system, the entire numerical processing is implemented in a MATLAB program (MATLAB © 2016) with a graphical user interface (GUI). Figure 5.3 and figure 5.4 present the layout of GUI in image mode and video mode. The flow charts of the software behind the GUI are displayed beside. Figure 5.3 shows the low frame imaging mode, which conducts reconstruction after taking a single frame of hologram and then takes next frame as a loop. Figure 5.4 shows the high frame rate video mode, which conducts reconstruction after taking multiple frames of holograms. In each mode, the interface is made up from four panels based on their properties, which are setting panel, control panel, performance panel and display panel, respectively A, B, C and D red frames. As shown in figure 5.3, the parameters can be set in A. And the full field of hologram, hologram within the region of interest (ROI), Fourier domain image (FFT image) and the phase reconstructions (red blood cells smear on a glass slide) are all displayed in C with two views: perspectives and top views.

To start running the program, a user first presses the Start Preview button in the control panel. On the upper left figure in display panel provide live preview the hologram from CCD camera. Users can optimize both gain and exposure time from the setting panel during preview. To select the ROI, the user first defines the size of the region by inputting Size: Height and Size: Width in the setting panel and presses the Crop button in control panel. A crosshair will appear along with the cursor. Users can place the crosshair on where is interested in the preview and click the left mouse button to fix the cropping. Then the selected ROI will be displayed right side of preview in the display panel. The objective options and tube lens usage are provided to users to choose in order to calculate the field of view.

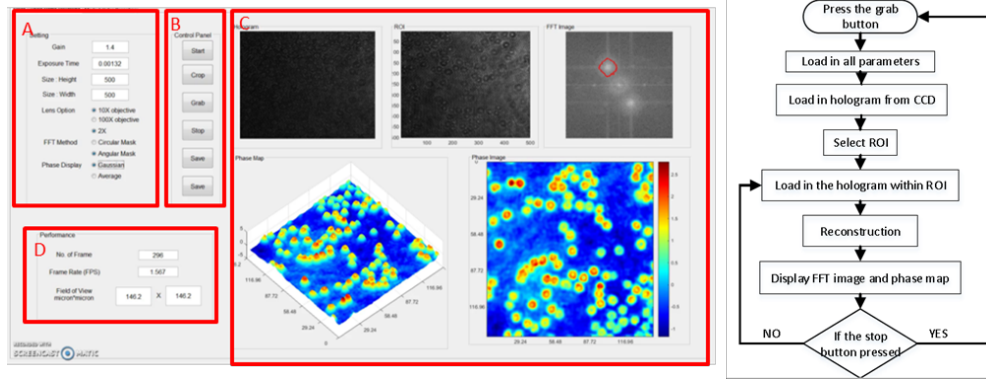


Figure 5.3 Layout of graphical user interface in image mode: red blood cells recorded with 10X NA 0.25 microscope objective. The flow chart of GUI design

To run the program under the imaging mode, the user needs to choose the spatial filtering method first. The default setting is the region recognition filtering introduced in the following chapter. User also can choose normal fixed circle window as spatial filtering method from the third pop-up menu in the setting panel. Similarly, users can choose if the phase result will be smoothed or not by selecting Gaussian Smooth or None from the last pop-up menu in the setting panel. Afterwards, users can press the Grab button in the control panel. The imaging processing will be executed to the hologram frame by frame and the spatial filtering image in frequency domain will be displayed in the third figure in the display panel. The phase reconstruction will be displayed on the second row of the display panel both in perspective view and top view. Meanwhile, the number of frames processed, frame rate, and the field of view will be shown in the performance panel.

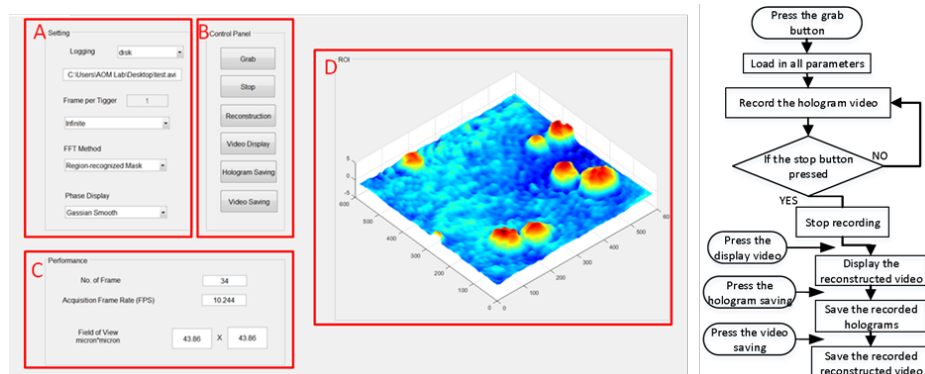


Figure 5.4 Layout of graphical user interface in video mode: yeast cells recorded with 10X, NA 0.25 microscope objective. The flow chart of GUI design

To run the program in the video mode, users can choose the spatial filtering method and smooth method same as in image mode. Users also can choose whether the video is logged in memory or the disk of the computer. For long terms experiments, i.e. time-lapse imaging, logging the video on the disk is recommended. Once the user selects the disk as logging method, in the first pop-up menu of the setting panel, a window explorer will appear to allow the user to choose the path and enter the video file name. After that, the user needs to choose how many frames per trigger form the second pop-up menu. The infinity option and the type-in option are provided for users to choose whether capturing the holograms continuously until pressing the Grab button or capturing a pre-set number of frames of holograms. A prompt window will appear to show the user the video has been recorded. The user then can press the Reconstruction button to execute the imaging processing on recorded holograms. The user will be notified by a prompt window showing the progress of the processing [Figure 5.5 (a) and (b)]. After processing has completed, the user can press the Video Display button to call out the Movie Player of MATLAB to display the reconstructed video [Figure 5.5 (c)].

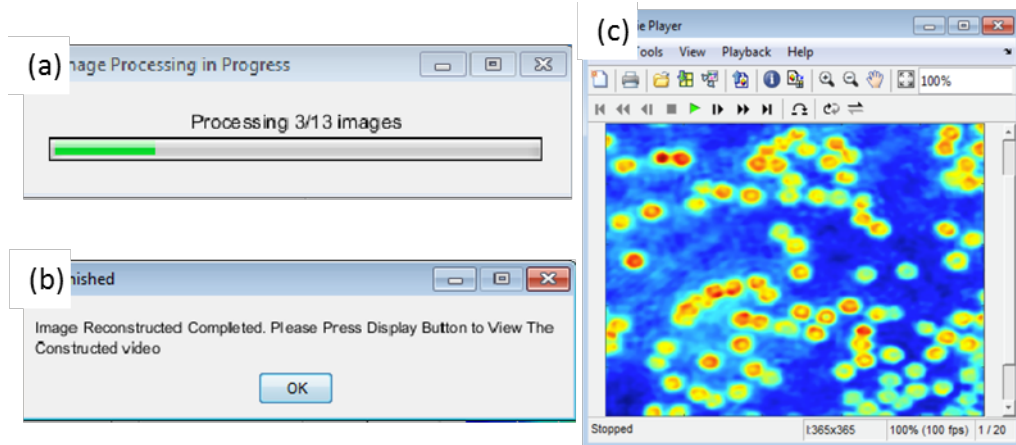


Figure 5.5 Popup windows during the operation. (a) and (b) are prompt windows during and the end of reconstruction processing. (c) is the Movie Player of the MATLAB to display the reconstructed video.

5.3 Automated holographic imaging

Adaptive spatial filtering offers fully automated phase reconstruction that can operate with a variety of samples and imaging conditions. When imaging

through an optically diffusing medium, the technique surpasses previous histogram thresholding techniques ⁶ without requiring any manual intervention.

5.3.1 Adaptive filtering with an iterative threshold for phase reconstruction

Here we propose an adaptive filtering process that is robust to different experimental conditions (fringe spacing, intensity of two arms of light, FOV, etc.). The method is built upon an iterative thresholding and region-based selection. Figure 5.6 shows the whole process. After a fast Fourier transform (FFT) of the recorded hologram [Fig.5.6 (a)], the phase shift is recorded in the spatial frequency in two symmetrical areas (+1 and -1 term) in the frequency domain (FFT image). The shapes and sizes of these two symmetrical areas can vary according to different imaging conditions. From figure 5.6 (b) to (f), the procedures are broken down into 3 steps:

- (1) Apply global threshold level (GTL)⁸ to the intensity of FFT hologram to get the binary image and then implement the region recognition process of it (*regionprops* function is used to provide three properties i.e. size of objects, centroid of objects in binary image and the box boundaries; *graythresh* MATLAB is used to get the GTL).

- (2) Increase the threshold level by one percent of GTL and repeat the first step until the number of regions reaches three (one for background intensity, one for the desired frequency component and one for the mirrored frequency component).

- (3) Use box boundary data from *regionprops* function and the binary image from the first step to get the right frequency component boundary and use it as a filtering window (additional Gaussian function is applied to smooth the edge of final filtering window).

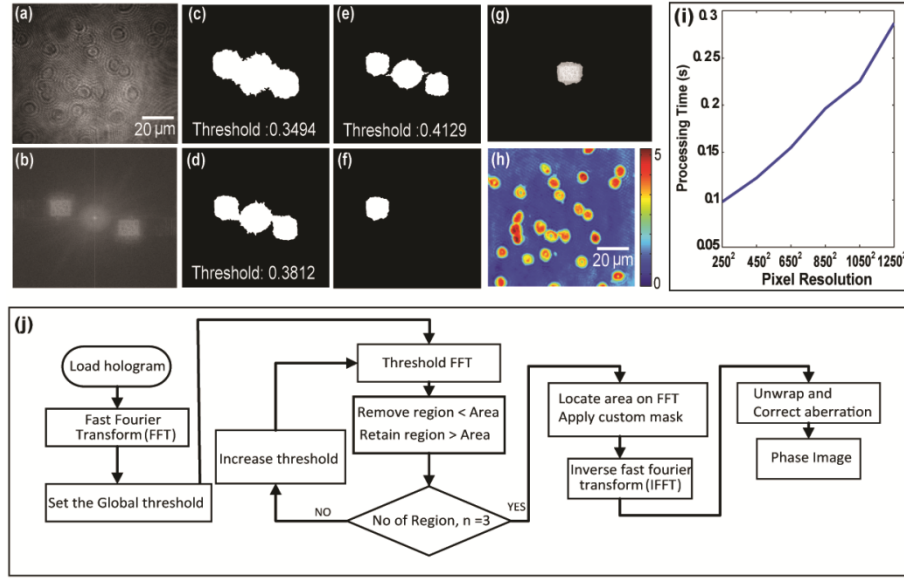


Figure 5.6 The region-recognition method. (a) Recorded hologram of RBCs (b) Spatial frequency of the hologram after fast Fourier transform (FFT). (c), (d) and (e), shows intermediate iterative thresholding process with incremental threshold level. (f) Optimised spatial filter window (g) Final filtered and centred spatial frequency area. (h) Reconstructed phase image. The colour bar gives the phase values by unit of radian. (i) Calculated time with different pixel size by this region-recognition method. (j) Overview of the flow chart of iterative thresholding and region-recognition program.

Once the appropriate spatial frequency is selected and shifted to the centre [Fig. 5.6(g)], an inverse FFT (iFFT) is performed to retrieve the final phase map of the sample [Fig. 5.6 (h)]. Processing time with different pixel size by this region-recognition method is provided in the plot figure 5.6 (i). Figure 5.6 (j) shows the flow chart of the iterative thresholding and region-recognition program.

5.3.2 Pattern recognition for cell segmentation

Analysis of cellular characteristics is often restricted to one of two scenarios: either averages of cell populations are examined (missing any heterogeneity that might be present within the population) or individual cells are studied (with the disadvantage of limited numbers and restricted *in-vivo* compatibility). For example, red blood cells (RBCs) are the most abundant cell type in the human body ⁹. Mutations in RBCs are very common and some have serious implications for the wellbeing of the carrier (e.g. Sick cell anemia, beta thalassemia, ovalocytosis ¹⁰). Moreover, infections (e.g. malaria, babesiosis ¹¹) and drugs (e.g. iodinated contrast media ¹²) can also influence the characteristics

of RBCs and the analysis of the characteristics is important for diagnostics, disease outcome and research. In order to provide our system with the flexible function to be competent for classification of RBC populations without the aid of labels rapidly by retrieving phase values for the individual, the region-recognition method in numerical reconstruction (chapter 5.2.1) was expanded toward characterization of RBCs, which is also critical for various biological cell studies.

Figure 5.7 illustrates the key steps (thresholding, perimeter location) used to extract individual RBCs. The first step is to read an image and apply a Gaussian filter for thresholding (Otsu's method ¹³). Next, holes are filled to remove small objects and extract perimeter pixels. The third step is to get the centre regions of individual cells and do the same processing of the second step with these centre regions. The fourth step is to inverse image intensity. The fifth step is to separate objects using watershed transform and measure the pixel areas of each object in the combined binary image. The final step is to label cell regions and remove false cell detections based on the pixel areas of objects. The phase value of each identified cell is saved into arrays for further analysis.

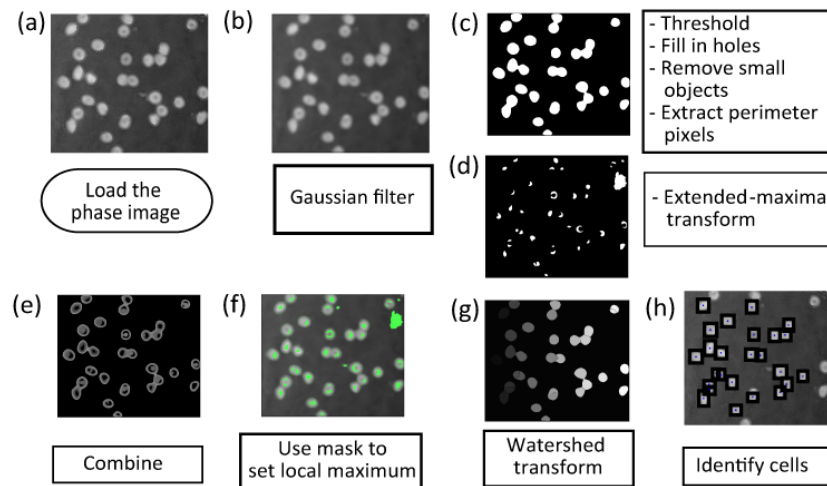


Figure 5.7 Region-recognition to extract individual RBCs. (a) load and (b) apply a Gaussian filter for thresholding. (c) voids are filled, small objects removed and perimeter pixels extracted. (d) maxima transform performed and apply step (e) combined the (c) and (d), (f) Invert image intensity and set local maximum in mask. (g) separate objects using watershed transform and (h) measure the pixel areas of each object in the combined binary image. (h) The final step is to label cell regions and remove false cell detections based on the pixel areas of objects.

5.3.3 Digital auto-focusing

Digital focusing is using computational method to retrieve the wave field at the sample plane. In order to make the system more automatic and less human operation required, autofocus function is added to the program. It was based on Fresnel–Kirchhoff integral that describes the propagation of a wave field. During the phase retrieval process [chapter 5.1.2], the complex matrix [Figure 5.1 (a)] obtained is the wave field of the object arm, indicated by $W_f(x, y)$ in equation (8). Digital focus can be achieved by propagating the wave field $W_f(x, y)$ by a certain distance d . The propagated wave field can be calculated by angular spectrum method¹⁴, shown below:

$$W_p = iFFT \left\{ FFT \left[W_f(x, y) \right] \times \exp[i\pi\lambda(\eta^2 + \xi^2)d] \right\} \quad (8)$$

In Eq. (8) λ is the laser wavelength, d represents the propagation distance.

The significance in auto-focusing function is to determine the propagation distance d automatically and intelligently. Hitherto, there are several methods to determine the focus plane, which are based on amplitude analysis¹⁵, intensity gradient¹⁶, local intensity variance¹⁷, Fresnelet-sparsity criterion¹⁸ and so on¹⁹⁻²¹. With the goal toward to biological study, most of the samples are pure phase objects²², in other words, the thin specimens, such as biological cells. Therefore, samples introduce little amplitude change due to the negligible absorption. In this case, we adopted the sharpness quantification^{20, 22} of the intensity image to determine the focus plane where the contrast of the intensity image is the smallest then the nearby plane. So the standard deviation of the amplitude matrix of the wave W_p is calculated to evaluate the degree of defocus, which should dwindle while the wavefront is propagating towards the focal plane. In order to approach the smallest contrast in the minimum trial of propagation calculation, instead of scanning a long range towards optical axis by a pre-set small distance interval, we applied Trisection Search Method²³⁻²⁴, a fast searching method for the extremum derived from Bisection Method²⁵, to find the focal plane within a fixed total travel distance.

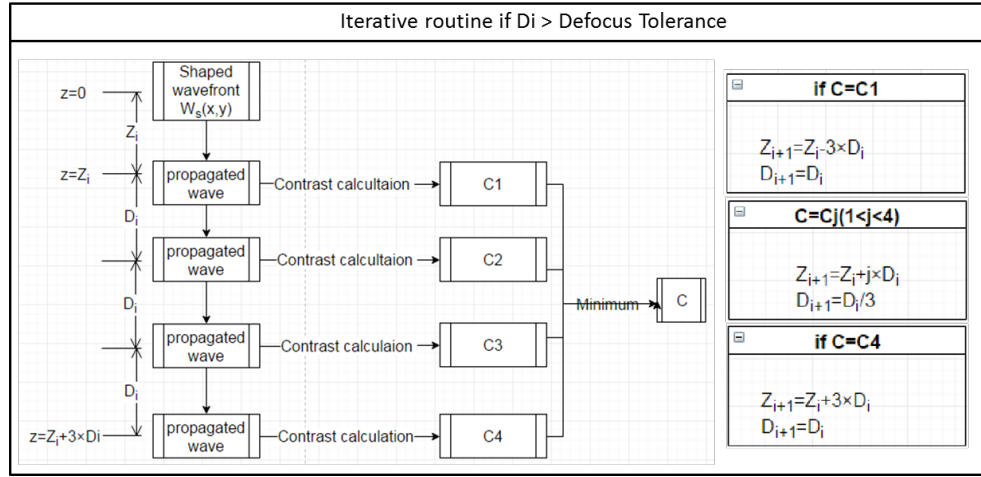


Figure 5.8 Flowchart of the digital focusing iterative routine. Z represents the coordinate along optical axis and i is the number of loops executed in the program. D_i is the propagation distance and C represents the contrast of the wave's amplitude distribution.

Figure 5.8 illustrates the detailed iterative routine of auto-focusing process. We define the total travel distance as 1.2 cm and the defocus tolerance as $0.5\mu\text{m}$ in our system. Firstly the original wavefront is postulated at initial point ($Z = 0$, Z is the coordinate towards the optical axis). In the first iteration, the original wavefront is propagated to four points along the optical axis, which locate at $Z_1 = -0.8\text{cm}$, $Z_2 = -0.4\text{cm}$, $Z_3 = 0$ and $Z_4 = 0.4\text{cm}$, separated by constant distance $D = 0.4\text{ cm}$. The standard deviations ($C1$, $C2$, $C3$, and $C4$) of wave amplitudes at these four points are calculated and compared. If the minimum of these four lies on $C1$ (or $C4$), the focal plane is at somewhere $Z < -0.4\text{cm}$ (or $Z > 0$). Then the propagation range is reset to a new set of four points immediately before $Z_2 = -0.4\text{cm}$ (or after $Z_3=0$), separated by the same distance D . If $C2$ (or $C3$) is the minimum, the focal plane must lie within the range $-0.8\text{cm} < Z < 0$ (or $-0.4\text{cm} < Z < 0.4\text{cm}$). Thus, the search range is narrowed down to $2 * D = 0.8\text{cm}$. Then the propagation range is shortened to four points within these two ranges and the distance between the new four points is two-thirds of the previous one. The process will be repeated until the distance becomes smaller than the defocus tolerance. In the last loop, the point with minimal contrast is identified as the final focal plane. Figure 5.9 shows an example of digital focusing of the RBCs images. Figure 5.9 (a) and (b) are the original phase image (color map in radian unit) and intensity image reconstructed from a defocused hologram. Figure 5.9 (c) and (d) are the digital focused results from the employed auto-focusing function.

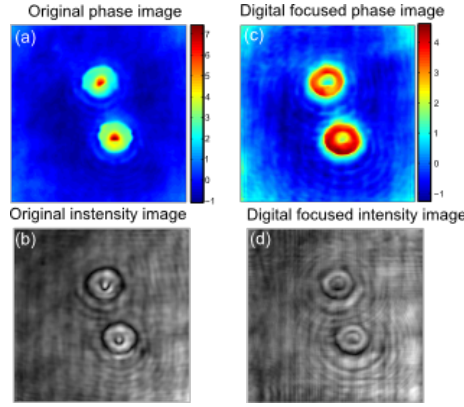


Figure 5.9 Result of the auto-focusing function. (a) and (b) are the original phase image and intensity image reconstructed from a defocused hologram. The (c) and (d) are the digital focused results phase image and intensity image. Colour map is in radian unit.

5.4 Calibration and performance

To calibrate the quantitative phase microscopy, it is necessary to use the specimen with known sizes and optical properties. The USAF resolution target card is a common example in intensity imaging systems. However, in phase microscopy, the phase only specimen is needed for standardizing the resolution and phase calibration. There are several works that proposed phase target specimens²⁶⁻²⁷ for standardizing quantitative phase imaging techniques, which often require electron lithography in order to fabricate the submicron structures. Alternative solutions are using microbeads to calibrate and characterize QPI systems²⁸⁻³¹. However, it should be noted that the microbeads have variance in size (typically smaller than 3 %). Figure 5.8 shows the result of profile of microspheres Polybead® Microspheres Polysciences, Inc.). The red lines are the reconstructed phase profiles from DHM system. Figure 5.10 a) demonstrates the phase profile of a 3 μm microsphere while b) shows the phase profile of a 45 μm microsphere. The blue lines are computed profiles.

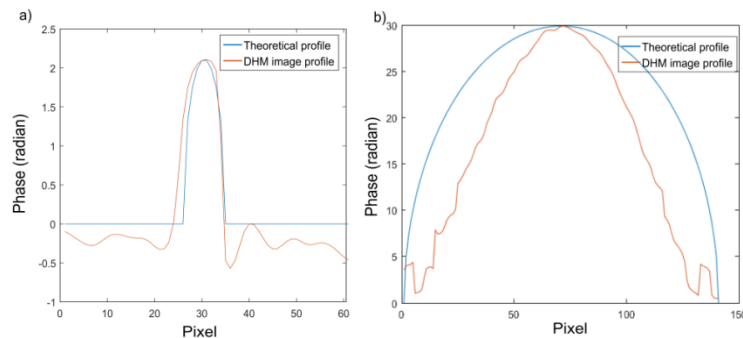


Figure 5.10 a) Phase profiles of a 3 μm microsphere. b) Phase profiles of a 45 μm microsphere. The red lines are the reconstructed phase profiles from DHM system. The unit is in radian. The blue lines are the theoretical profiles of the spheres based on the projection of a sphere. The material of the microsphere is polystyrene with refractive index of 1.587.

The performance of the program is characterized by the frame rate of image capturing and reconstruction. In image mode, the frame rate is defined by how many frames are captured and processed in one second. In video mode, the frame rate is how many frames are recorded in the video per one second. Table 5.1 lists the program performances for images of different sizes.

Image size (pixel ²)	Camera Acquisition (FPS)	Imaging mode Acquisition (FPS)	Video mode Acquisition (FPS)
256*256	22.5	1.65	19.6
384*384	16.2	1	14
512*512	12.6	0.66	11.7
768*768	8.8	0.33	8
1024*1024	6.6	0.2	6
1536*1536	5.1	0.08	4.5
2048*2048	3.5	0.046	3.29

Table 5. 1 Frame rates of two modes for different image sizes

Here we demonstrate the performance of the proposed DHM system on imaging the scattering medium. There have been previous techniques⁶⁻⁷ aimed at providing adaptive filtering methods by using histogram analysis to settle the appropriate threshold. However, these methods entail a series of steps that require manual settings or prior knowledge of the frequency distribution. For example, the first procedure in pervious technique [18] is to apply a negative Laplacian³² to suppress zero order in FFT hologram, which requires distance between first and zero order frequency components to be known prior or well-defined in the FFT hologram. Intuitively, the approach may not operate well when positions of the spatial frequencies change (i.e. different samples and interference fringes on the CCD camera or that the samples has large optical scattering).

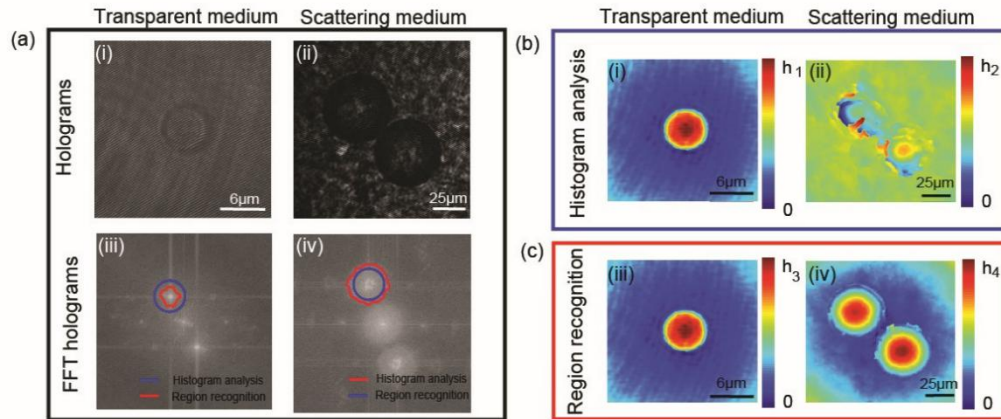


Figure 5.11 Comparison of histogram analysis filtering technique ⁶ with region-recognition method under different sample conditions. (a), (i) and (ii) are the holograms of a 6 μm diameter microsphere through water and two 50 μm diameter microspheres through opaque peptide hydrogel. (iii) and (iv) are the FFT holograms of (i) and (ii) respectively. In (iii) and (iv), the red outlines represent the best filters from region recognition method and blue circles represent pre-cropping the area for histogram analysis. Reconstructed phase image with histogram analysis are shown in (b) and region recognition method (c). Colour bar $h_1 = 6.7 \mu\text{m}$, Colour bar $h_2 = 50 \mu\text{m}$, Colour bar $h_3 = 6.7 \mu\text{m}$, Colour bar $h_4 = 50 \mu\text{m}$

The caveat of previous histogram analysis technique ⁶ is the requirement to first manually pre-crop the area containing the first order frequency. The histogram of the area would then be analysed accordingly (shown in more detail in Appendix 2). The drawback of using such manual pre-cropping process is that it influences the quality of the final reconstruction from sample to sample. We illustrate this point by directly applying the same pre-cropping and histogram analysis to retrieve phase images of polymer microspheres in transparent medium into a scattering medium. The reconstructed phase is then compared with the iterative threshold technique. The purpose of this study is to prepare an automated DHM system that is suitable to study living cells developing into a living tissue. In thick samples, such as thick tissue culture, the effect of scattering is more pronounced and hence the reconstruction becomes more complex due to higher frequency noises. We therefore test the thresholding process on their performance sample with thick ($\sim 100 \mu\text{m}$) optically scattering biological-relevant matrix (peptide-hydrogel). The scattering medium (Appendix 2 Figure A2.1) is prepared with 50 μm polymer microspheres (Duke Scientific) deposited onto a thin film of

biological hydrogel matrix peptide hydrogel (Fmoc-FRGDF) ³³. The peptide hydrogel has been used in thick cell growth culture for neuronal growth. The hologram of a polymer microsphere in water, figure 5.11 (a) (i), is significantly different from the hologram of hydrogel matrix [Figure 5.11 (a) (ii)], as the latter displays a large background scattering with significant increase in opacity. The scattering sample generates additional spatial frequency content that “blurs” the boundaries and “extends” the dimensions of three spatial frequencies components [Figure 5.11 (a) (iv)]. That adds complexity to precise selection of first order frequency content for phase retrieval process. The results of the reconstructed phase maps from the histogram analysis technique. Figure 5.11 (b), and our approach, figure 5.11 (c), show that the histogram analysis approach works in a standard transparent medium figure 5.11 (b) (i), but clearly fails to extract the appropriate spatial frequency in the scattering medium as shown in figure 5.11 (b) (ii). The iterative method works for both samples as shown in figure 5.11 (c) (i) and (ii). The comparison indicates that the filtering process is complicated by the broad distribution of spatial frequency. Previous histogram analysis technique failed to obtain the desired reconstruction. While our approach, iterative thresholding and region recognition [Chapter 5.3.1], successfully retrieved optimal shape of the filtering windows [blue curves in Figure 5.11 (a) (iii) and (iv)] and obtained accurate reconstruction of the microsphere through a diffusive medium [Figure 5.11 (c)]. A more detailed breakdown of the steps of both the histogram analysis technique and threshold increment determination are shown in Appendix A2. We also include another histogram analysis ⁷ in Appendix A2 that also fails to retrieve the spatial frequency of scattering medium.

The performance of proposed adaptive spatial filtering method and overall for phase reconstruction of installed digital holographic microscopy system is tested by various samples, including the murine primary cortical neurons, neural stem cells in a hydrogel culture ³⁴ (highly scattering medium), a red blood cell on smear and 6 μm diameter polystyrene microsphere (Polybead® Microspheres Polysciences, Inc.).

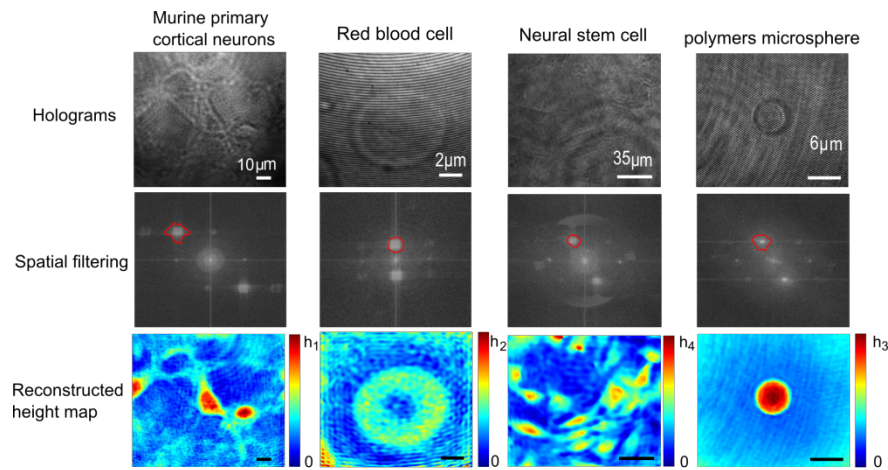


Figure 5.12 Examples that different samples and imaging conditions as well as the reconstructions from DHM system.

5.5 Conclusion

DHM relies heavily on accurate execution of numerical processes to reconstruct the phase of the biological sample. While off-axis DHM technique is perhaps the fastest holographic imaging technique, the accurate filtering of the spatial frequency can affect the final reconstructed image. We demonstrated that the combination of region-recognition and iterative thresholding conducted in a single loop can aid in selecting the optimal spatial frequency components with marginal delay (~ 0.1 to 0.3 s). The effectiveness of our process is illustrated in the comparison study where the iterative approach clearly identifies reconstructed microspheres embedded in opaque hydrogel matrix ($\sim 100 \mu\text{m}$) has addressed the use of the technique in thick samples. More importantly, as compared with past techniques, we showed that this technique can operate well in both transparent and turbid system, thus providing potential flexibility for imaging live cell activities³⁵. In the next step, we will aim to conduct live cell imaging in confluent cell cultures that will aid in the study of cellular process in engineered tissue.

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Chapter 6 Non-invasive deformability measurement of live parasite infected RBCs using digital holographic microscopy

Part of the work in this chapter has been presented in the conference:

He X, Maier AG, Lee WM. Mapping the progression of malaria infected erythrocytes with holographic microscopy. InConference on Lasers and Electro-Optics/Pacific Rim 2015 Aug 24 (p. 27P_73). Optical Society of America.

Contributions of others:

Sample preparation related to RBCs was done by a PhD student, Mrinalini Pratap supervised by Maier AG.

6.1 Malaria

Malaria is a life-threatening disease caused by Plasmodium parasite protozoans that are transmitted to people through the bites of infected female Anopheles mosquitoes. At the end of 2004, 107 countries and territories had areas at risk of malaria transmission ¹. In the wild, there are 5 parasite species that cause malaria in humans, and 2 of these species – *P. falciparum* and *P. vivax* – pose the greatest threat. *P. falciparum* is the most prevalent malaria parasite on the African continent. *P. vivax* has a wider distribution than *P. falciparum*, and predominates in many countries outside of Africa. Falciparum malaria causes more than 1 million deaths each year. In this chapter, the non-invasive morphology study of *P. falciparum* infected red blood cells (described as malaria parasites infected RBCs or infected RBCs in the following content) was conducted by digital holographic microscopy with an adhesion assay measurement.

6.1.1 Malaria infection

Red blood cells (RBCs) infected with the malaria parasite undergo remarkable structural, biochemical and mechanical modifications. One of the major structural changes is the formation of parasitophorous vacuoles surrounding the growing parasite inside their host RBCs ², which induces nanoscale protrusions on the membrane surface. The mechanical modifications are the loss of RBCs' deformability ³ and the increase of RBCs' cytoadherence ⁴. These

modifications give rise to vital organ dysfunction due to the sequestration of RBCs within microvasculature in the later stages of parasite development. However, the complete mechanism and the pathogenesis of the alteration are still poorly understood ⁴⁻⁵. The parasite life cycles and infected RBC membrane surface alteration are illustrated in figure 5.1. Infection starts from a blood meal when a mosquito, carrying the protozoons, releases sporozoites into human skin. The sporozoites travel to the liver and invade into the hepatocytes, a main component of parenchymal tissue of the liver, where they divide for around 10 days and differentiate into merozoites subsequently. The merozoites are then released into the bloodstream and enter the RBCs. During the development in RBCs, the parasite starts with the ring stage (0-24hours), followed by trophozoite stage (24-36hours) with DNA replication and ultimately the schizont stage (36-48hours) when multiple merozoites are formed and lead to rupture of the RBCs. Figure 5.1.c shows the export of parasite proteins during the parasite development inside the RBCs. It starts from endoplasmic reticulum into the parasitophorous vacuole (PV) by a secretory pathway. Then the exported proteins get through the parasitophorous vacuolar membrane (PVM) and reach the membrane of the RBCs. Eventually, the exported proteins including *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) are part of Maurer's clefts and the knobs ⁶⁻⁷, which contributes the abnormality of infected RBCs ⁸⁻⁹.

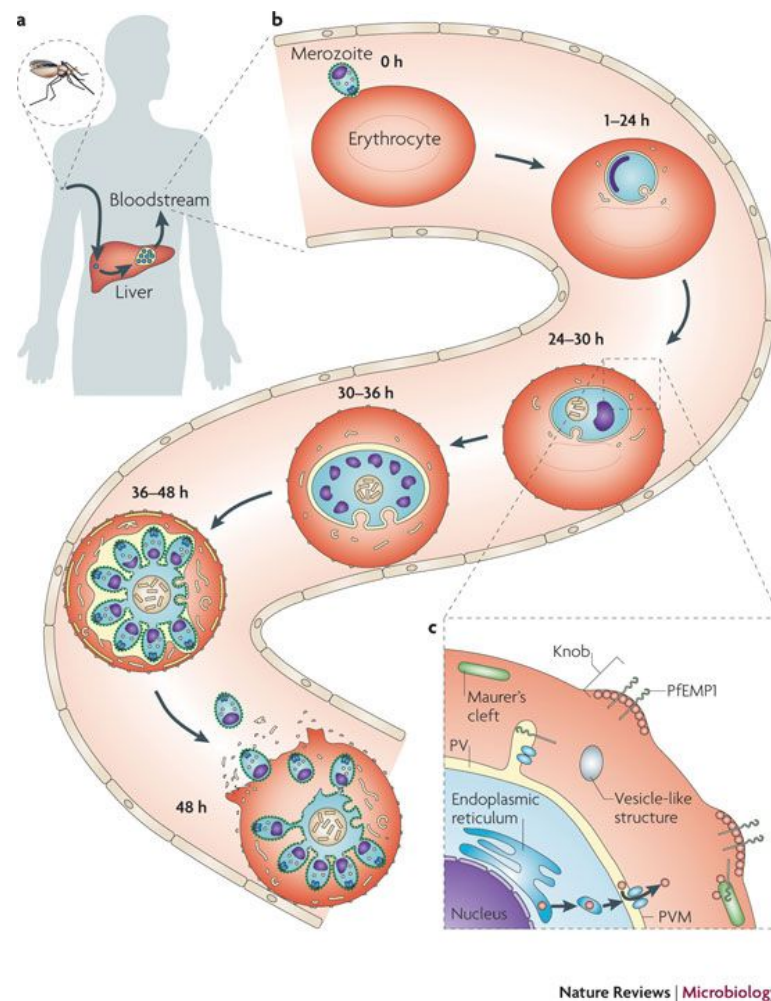


Figure 6.1 *Plasmodium falciparum* life cycle, reprinted with permission from Goldberg et.al ¹⁰ © 2010, Springer Nature. a, Infection. b, Parasite development inside RBCs, Infected RBCs membrane alteration by proteins export dominated by the parasites. Parasitophorous vacuole (PV). Parasitophorous vacuolar membrane (PVM). Plasmodium falciparum erythrocyte membrane protein 1 (PfEMP1).

6.1.2 Imaging the malaria parasite infected RBCs

There are a variety of practical techniques aiming for diagnosis of malaria infection ¹¹⁻¹². However, the early and fast diagnosis with a minimal dose of blood is still challenging. Some studies are targeted at using microfluidic methods to separate infected RBCs from blood, which ideally help improving the diagnosis by increasing the infected RBCs density ¹³⁻¹⁵. Directly detecting the infected RBCs by staining nuclei of the parasites was the traditional diagnosis approach. But it lacks a clear prognosis of the stages of infection. The morphology and cellular structure of the infected RBCs change significantly with the development

of the malaria parasites. Although the alteration leads to the critical morbidity, the mechanism of it is intricate and still not fully revealed¹⁶. Different imaging techniques have been applied for static and dynamic measurements of infected RBCs. For example, atomic force microscopy (AFM) was used to precisely measure the surface morphology of individual infected RBCs in different stages (separated by controlling infection timing)¹⁷. Fluorescence microscopy is commonly used to identify malaria parasites by staining the nucleus of the parasites¹⁸⁻¹⁹ or observing morphology by labeling the cytoplasm of the RBCs. Esposito *et al.* conducted a comprehensive investigation on volume and membrane area changes in infected RBCs using fluorescence confocal microscopy²⁰. Recently, the quantitative phase microscopy has merged into the powerful imaging tool for cell pathophysiology study²¹ and it is efficient for conducting the dynamic measurement²², especially when combined with the microfluidic devices. Moreover, Kang *et.al* employed a multimodal microscopy system to provide both chemical and morphological information of infected RBCs²³. There are also some simulation methods to compute the behavior and the force of the infected RBCs undergo in the microfluidic channels²⁴.

Among different investigations, the techniques used to deform the cells in a controlled way and the imaging techniques for extracting the useful information (molecule specificity, volume, two or three-dimensional (3D) shape changes and so on) are carefully chosen based on the experimental condition and design. Some studies were more focused on the manipulation of the RBCs²⁵⁻²⁷ while others paid more attention to the analysis the detailed morphology^{17, 28-29}. In this chapter, we shall target the development of an adhesion assay to quantify deformability of RBCs using QPM imaging.

6.2 Materials and methods

This section demonstrates the methods of the experiment and sample preparation.

6.2.1 Methods outline

The off-axis digital holographic microscopy (DHM) system, introduced in chapter 5, was used as the imaging tool offering two and a half dimensional

(2.5D) images/videos of unstained RBCs. In this study, we investigated the infected RBCs with multiple measurements. First, the blood smear samples, both healthy and infected RBCs, were imaged by DHM. The obtained volumetric images, which stand for the static morphologies, were analyzed quantitatively. Secondly, the dynamic deformability measurement of the RBCs was conducted in a simple microfluidic channel with RBCs adhered on the bottom of the channel (adhesion protocol can be referred in chapter 6.2.2). The changing shear stress controlled by a pressure pump (model) was used to deform the RBCs. Three types of live RBCs were investigated totally, the uninfected RBCs, the artificial inelastic RBCs, and infected RBCs. Table 6.1 demonstrates the experimental setting.

Sample	Morphology (static)	Deformability (dynamic)
Uninfected RBCs	Blood smears	RBCs adhered assay in microchannel
Inelastic RBCs		
Infected RBCs	Blood smears	

Table 6. 1 Experimental setting.

6.2.2 Double container imaging chamber for infected RBCs in microfluidics

For live infected RBCs, the malaria parasites are living inside the RBCs (small portion of them are in the solution outside the RBCs that needs to be handled in a sterile environment. In order to handle those samples in a non-sterile environment (non-Physical Containment 2 Facilities, (non-PC2)), we proposed the use of a double container that stores sample for biosecurity control (in accordance to Biological Safety Policy of Australian National University and Australian Government Department of Health). We especially designed a secured transparent double container to hold samples with microfluidic capabilities. Figure 6.2.a) shows the DHM system and b) presents the photo of the container. Figure 6.2.c) demonstrates a cross section of side view of the design and equipped pressure pump system (AF1 dual-microfluidic and vacuum pump, Elveflow). Two filter discs (0.2 μm , Sterile, Thermo Fisher Scientific) were used at the entrance to prevent infected RBCs and parasites from escaping from the container. A microfluidic reservoir (50 mL Falcon Tube 1/1 port, Elveflow) was used for the air passage and to prevent any liquid from going to the pressure pump when there

is negative pressure applied. To fabricate the container, acrylic sheets were used as the material for good optical transparency and isolation. The sheets were laser cut into designed pieces and assembled by an adhesive (cyanoacrylates). The bottom part, where the microfluidic chip and sample reservoirs sit, can be removed prior to the experiment for convenient installation.

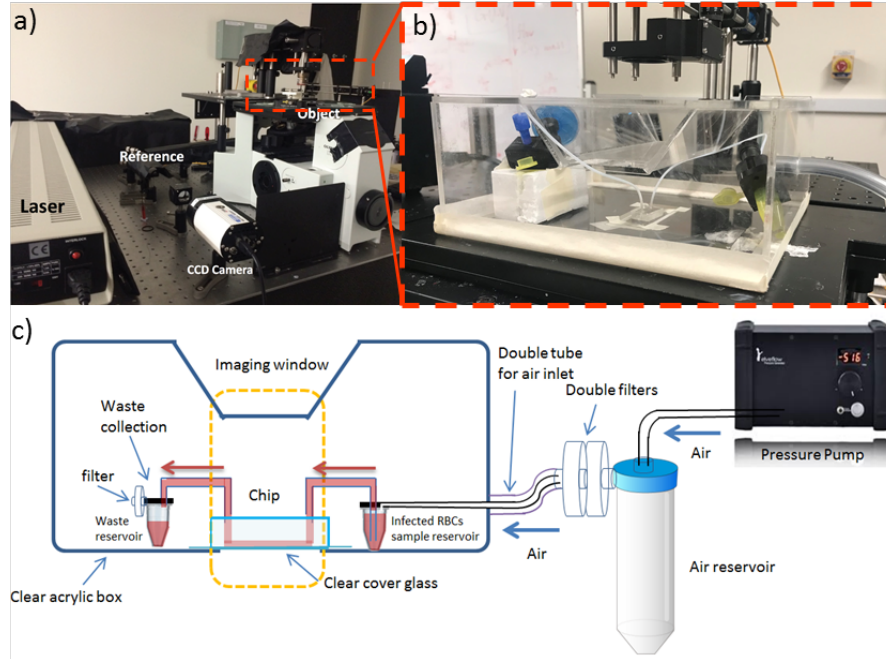


Figure 6.2 DHM Imaging setup with double container imaging chamber for live infected RBCs experiments. (a) Photo of DHM system (b) Photo of double container on microscopy stage. (c) Diagram of design of double container and pressure pump system.

6.2.3 Sample preparation

In this section, sample preparation procedures are listed out, which include fixed RBCs sample (blood smears), artificially inelastic RBCs, and live infected RBCs as well as live healthy RBCs. Fresh O+ blood were donated from the Red Blood Cross, Canberra (ACT). And the laboratory strain (CS2) is original from reference ³⁰.

6.2.3.1 Blood smears

Glass slides (1.2mm, Livingstone) were cleaned prior to blood smears. Slides were dipped in acetone, rinsed with deionised water before being dipped in methanol and rinsed again with deionised water. Slides were dried with nitrogen gas before preparing blood smears. The smears were fixed with methanol (100% or absolute) and dried completely before imaging.

6.2.3.2 Artificially inelastic RBCs

Uninfected red blood cells were artificially stiffened to varying degrees by different concentration of glutaraldehyde. Fresh 25% glutaraldehyde (Electron Microscopy Sciences) was diluted through serial dilution down to a concentration of 0.01, 0.02% (v/v) in Phosphate-buffered saline (PBS). Uninfected red blood cells in supplemented RPMI at 4% haematocrit (HCT) were centrifuged at 500 xg for 5 minutes at room temperature to pellet the cells. Later an equivalent amount of diluted glutaraldehyde was added. The sample was incubated for 10 minutes at room temperature. The glutaraldehyde solution was then removed from the cells by centrifugation at 500 xg for five minutes at room temperature and rinsed in PBS thrice before resuspending in PBS to a 4% HCT.

6.2.3.3 Malaria infected RBCs

Continuous culture of parasites in vitro:

Plasmodium falciparum (described as parasites in the following text) were incubated and grown in RPMI 1640 medium (Gibco) which was supplemented with GlutaMAXTM II 0.38% (w/v) AlbuMAXTM II (Invitrogen), 2.5% pooled heat inactivated human serum, 20 µg/mL Gentamicin (Gibco), 480µM hypoxanthine, 10mM D-glucose and 4% haematocrit of O⁺ blood. Cultures were maintained in 10ml or 30ml culture dishes kept in a hypoxic gaseous environment of 1% O₂, 5% CO₂, 94% N₂ at 37°C. A continuous culture of parasites was maintained by diluting the parasitemia to 0.2% and feeding with fresh blood and media every 48 hours. In the following text, the parasite culture refers to the mix of parasite and fresh blood and RPMI 1640 media.

Magnet purification of parasites:

Although in the same parasite culture, the stage of infection among all the RBCs population is not the same (mix of the trophozoites stage, schizont stage, ring stage and uninfected state). The purification of the parasites needs to be conducted to provide relatively uniform parasite culture. Parasite in trophozoites and later stage were purified from the ring stage and uninfected RBCs through an iron wool column placed in a magnetic field as described in the paper ³¹. Purified parasites culture was recovered in supplemented RPMI 1640 media for an hour at 37°C.

Synchronisation of parasites via sorbitol lysis:

Ring stage parasite culture was obtained via sorbitol lysis³² 18 hours post infection, which selectively lyses mature stage parasites. Cell pellet was obtained from the mixed culture later and was incubated with 5% D-sorbitol for 10 minutes and subsequently recovered in supplemented RPMI.

6.2.3.4 Diluted RBCs PBS solution for microfluidic chip infusion

The RBCs samples were diluted in PBS solution at a ratio of 1:10 to reach the lower concentration of the RBCs in solution. The infusion was taken immediately after the dilution. The procedure is aimed to avoid dense RBCs population over the channels, and to make sure there is no overlap RBCs in the microscopic FOV.

6.2.3.5 Microfluidic chip fabrication and surface modification

The PDMS microfluidic devices were fabricated by soft lithography process described earlier in chapter 4. The molded PDMS chunk was bonded on the coverslip to serve as the microfluidic chip, which allows short working distance objective lens to be used to image the channel inside. The dimension of the microchannel is at 100 μm width and 25 μm high. The channel inside was treated before the experiment, in order to provide the adhesive surface for RBCs stick to. Firstly, pure ethanol was flowed into the channel for 10 mins. 4% APES (3-Aminopropyltriethoxysilane) in ethanol serves as the adhesion promoter³³ was perfused for 15 min. after that, the 50-100 $\mu\text{g/ml}$ Con-A solution (Concanavalin A, Sigma) was filled up with the channel for 2 hours room temperature (or overnight 2°C). The microfluidic channel was rinsed with PBS slowly (7.5 $\mu\text{l/min}$) before experiments.

6.3 Measurement and results

6.3.1 Static morphology

The static morphology of uninfected RBCs and infected RBCs (trophozoites stage) were observed by DHM with the blood smears. Previous studies have shown that infected RBCs possess a distinctly different morphological deformation when compared to the uninfected RBCs³⁴, which is

consistent with our observation. The distribution of phase values represents the height variation of malaria parasite infected and uninfected RBCs. Figures 6.3 (a) and (b) are the Scanning electron microscopy (SEM) pictures of malaria parasite infected and uninfected RBCs respectively, while figure 6.3 (c) and (d) are the phase reconstructions of an infected and an uninfected RBCs, which were taken and reconstructed from our system with high magnification objective lenses (100X, N.A 1.25). The colour bar shows phase values in the unit of radians. From the phase maps and the SEM pictures, infected RBC has characteristic anomalous shape while uninfected RBC shows a normal biconcave shape.

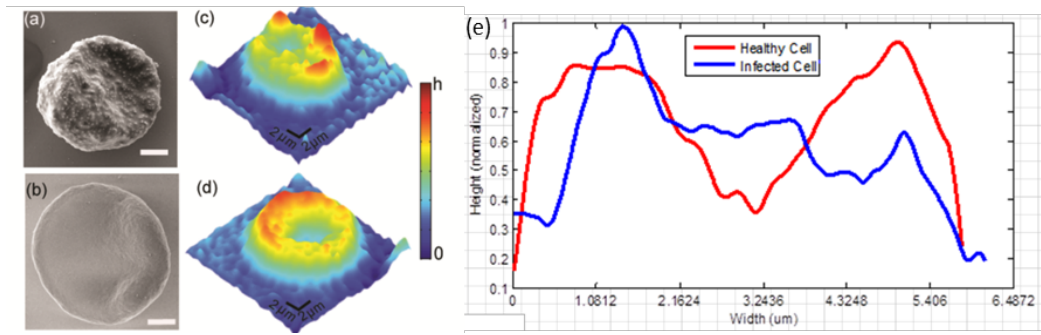


Figure 6.3 Example images of red blood cells taken with scanning electron microscope (a), (b) and DHM (c), (d). Infected (a), (c) and non-infected (b) and (d). , (e) Cross section profiles of infected (blue) and healthy (red) RBCs.

In order to analyze the morphological differences between infected and uninfected RBCs, the phase value over the cell surfaces is selected out to plot the histograms. The black dots in figure 6.4 (a), (b) are the histograms of normalized phase values over RBCs shown as thumbnails respectively. The histogram indicates how heights of a cell distribute over its surface. The solid curves in Figs. 6.4 (a), (b) are the Gaussian fittings of the histograms. Quantification of the phase is inferred from the full width at half-maximum (FWHM) of the fitted curve of the phase distribution provides an indication of the spatial distribution of the phase (height of a cell) variation over an entire cell as shown in figure 6.4 (a), (b) shaded region. Figure 6.4 (c) shows the distribution of FWHMs of the spatial distribution of the phases on 12 infected and 12 uninfected RBCs. The trends indicate a marked difference between infected and uninfected RBCs. The infected RBCs most likely have lower FWHMs (the histogram is more centralized), which means the differences in cell height are not major. Therefore, the blue line in figure 6.4 (c) is the

histogram of FWHMs of the infected RBCs. It shows most of infected RBCs have FWHMs at below 0.1, while uninfected cells (red line) tend to have higher FWHMs (0.25 to 0.3). This shift indicates that the height of uninfected RBCs changes largely spatially. In other words, the uninfected RBCs have broader height (phase) distribution. It can be explained by the fact that the uninfected RBCs have a thinner central height. Therefore, the height difference between the centre and the periphery is larger than that of infected RBCs where the volume of the centre part is occupied by the parasite.

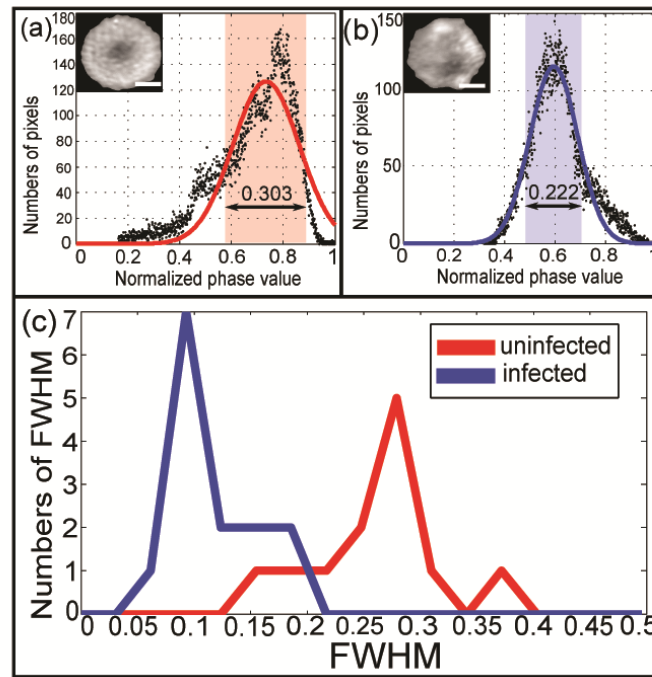


Figure 6.4 Phase distribution analysis of malaria infected RBCs versus uninfected RBCs. (a) Histogram of phase value over an infected RBC surface shown in inset $2\mu\text{m}$ scale bar. (b) Histogram of phase value over an uninfected RBC surface, which is shown as thumbnail with $2\mu\text{m}$ scale bar. (c) Histograms of FWHMs of 12 uninfected RBCs' phase distribution (red) and the histogram of FWHMs of 12 infected RBCs' phase distribution (blue). This shift between two histograms indicates that uninfected RBCs have broader height (phase) distribution.

6.3.2 Measuring deformability by adhesion assay

One of the key disadvantages of blood smear investigation is the inability to quantify mechanical responses from live cells which limits the amount of information that can be extracted during live imaging. In order to provide a

reliable technique that can quantify iRBC without resorting to a complex microfluidic system, we developed a surface adhesion assay.

6.3.2.1 Hydrodynamic stretching methods

Diluted RBCs PBS solution was infused into the microchannel at 55 mbar pressure which was 24 $\mu\text{l}/\text{min}$ flow rate measured by Bronkhorst Coriolis flow sensor (BS1, Elveflow). Later the pressure was reduced to 5 mbar ($0 \pm 0.2 \mu\text{l}/\text{min}$) to keep the RBCs stationary and settle down in the microchannel for 5 min. The process was aimed to let RBCs bind on the Con-A coated microchannel (stationary duration time was tested repeatedly to reach robust binding under experimental shear conditions). After 5 min standing, the microfluidic chip was installed in the double container with a reservoir filled with PBS. Flowing on that, the double container was placed on the DHM imaging stage for experiment observation. During the experiment, RBCs dynamic deformation was induced by hydrodynamic stretching. Since the bottom side of RBC was tightly bound on the Con-A coating and the upper side of RBCs was stretched by fluidic shear. The fluidic shear produced by flow was controlled by a pressure pump through two modes: manual mode and program mode. The process of determining the experimental input pressure setting was introduced in chapter 6.3.2.3. Figure 6.5 shows the schematic diagram of a microfluidic chip and adhesion assay.

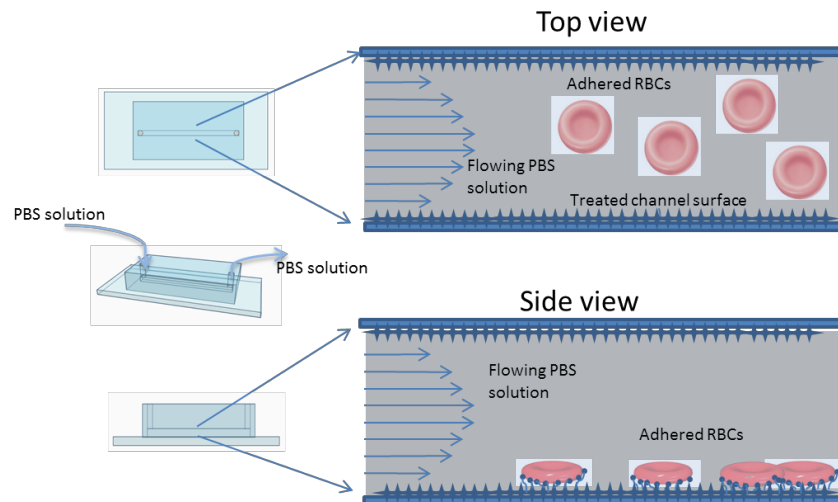


Figure 6.5 Schematic diagram of adhesion assay

6.3.2.2 Quantifying Deformation: Centroid tracking

A sequence of RBCs volumetric images $H(x,y,t)$ was constructed by DHM system, which was indicated by the height value at coordinate (x,y) at the corresponding time point t . One of the most frequently used morphological quantifications of a two-dimensional object is calculating the degree of circularity or elasticity³⁵. Since the volumetric images obtained in our system were the height maps, which added another dimension (z) to our knowledge. The quantification can be also performed in full 3D space. The centroid, representing the mass spatial distribution of a 3D object, was calculated from the volumetric image from QPM system. And the changing of centroid over time quantifies the deformation. The centroid calculation refers to the equations below:

$$v(x,y,t) = h(x,y,t) * A_p \quad (1)$$

$$V(t) = \sum_{x=1,y=1}^{X,Y} h(x,y,t) * A_p \quad (2)$$

$$C_x(t) = \frac{\iiint v(x,y,t) * x * dx dy dz}{V(t)} \quad (3)$$

$$C_y(t) = \frac{\iiint v(x,y,t) * y * dx dy dz}{V(t)} \quad (4)$$

$$C_z(t) = \frac{\iiint v(x,y,t) * z * dx dy dz}{V(t)} \quad (5)$$

where $h(x,y,t)$ is a sequence of a single RBC volumetric image, x and y are coordinates of the image and t is the time point when the image was taken. The single RBCs volumetric image was obtained from full-field image $H(x,y,t)$ by the pattern recognition method introduced at chapter 5.3.2. A_p is the area of a single pixel in the image, calibrated with an object of a known size (1951 USAF Glass Slide Resolution Target Card). Eq.1 is for calculating the volume of local point (x,y) at time point t . $V(t)$ is the total volume of the RBC, calculated from Eq.2. C_x , C_y , C_z demonstrate the position of the centroid of the RBC in x , y and z coordinates at time point t .

A preliminary test was done on healthy RBCs and artificially inelastic RBCs. The fluidic flow was controlled manually. The purpose of the preliminary test was to search the extreme pressure that the binding of RBCs to Con-A coated substrate could tolerate. Figure 6.6 shows the one episode of a healthy RBC and

an artificially inelastic RBCs (0.02% glutaraldehyde) in the same microchannel under the fluidic shear. On the left side of the figure, snap shot of healthy RBC and artificially inelastic RBC in top view and 3D view is presented. The three line plots on the right demonstrate the position of the centroid of the cell during the time in x, y and z coordinates. The flow direction was along y coordinate. The inelastic RBC (red line) shows completely no deformation while the healthy RBC (blue line) represents the highly flexible stretching (around 0.2 μm in z direction, 1.5 μm in x direction and 5 μm in y direction shift of centroid).

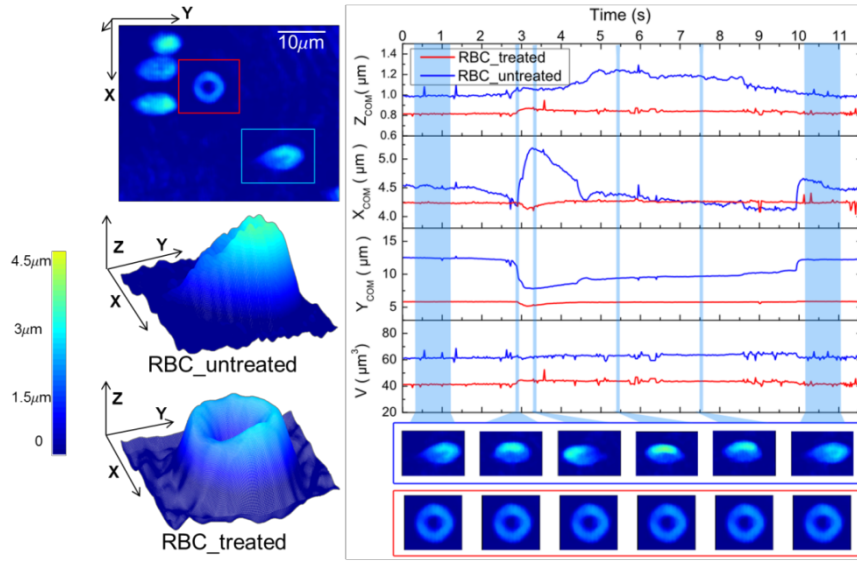


Figure 6.6 Deformation of a healthy RBC and an artificially inelastic RBCs (0.02% glutaraldehyde) in the microchannel.

During the test, 5 mbar input pressure was found to be able to compensate the inlet and outlet pressure difference and keep the flow stationary. The Con-A bounding of inelastic RBCs was stable within -45 mbar to 50 mbar input pressure range (-45/50 mbar for a half minute and -25/30 mbar for more than 5 mins without migration). Healthy RBCs and artificially inelastic RBCs can bear the input pressure -45/50 mbar more than 5 mins without migration. Therefore, the experimental input pressure was chosen from range of -20 mbar to 25 mbar to ensure investigated RBCs tightly bound to the Con-A coating.

6.3.2.3 Testing and results

First, the artificially inelastic RBCs were investigated with sine wave flow (0.2 HZ, 5 mbar to 25 mbar, -0 to 12.011 $\mu\text{l}/\text{min}$) which is indicated by the dash grey line in figure 6.7 (a). The deformation of cells was quantified by the centroid position changing along the direction of the flow (unit was normalized). The artificially inelastic RBCs are 0.01% and 0.02% glutaraldehyde treated RBCs (tRBCs, chapter 6.2.3.2). The 0.02% tRBCs (averaged with 10 RBCs deformation data) show completely no deformation [yellow line in figure 6.7 (a)], which is the same with the test before [Figure 6.7]. The 0.01% tRBCs [red line in figure 6.7 (a) (averaged with 5 RBCs deformation data)] shows similar trend with the untreated healthy RBCs [blue line in figure 6.7 (a), (averaged with 5 RBCs deformation data)]. They were stretched immediately when the flow started increasing due to the high deformability. In order to have a constant gradient of flow rate profile, triangular wave function was chosen to test malaria infected RBCs for linear increasing and decreasing of the flow rate (0.2 HZ, 5 mbar to 25 mbar, -0 to 12.011 $\mu\text{l}/\text{min}$ measured by flow meter). Figure 6.7 (c) shows the results of infected RBCs and healthy RBCs deforming under the fluidic in a triangular wave [the dash grey line in figure 6.7 (c)]. The red line represents the deformation of healthy RBCs (averaged with 13 healthy RBCs deformation lines) and the red line shows the deformation of infected RBCs (averaged with 4 infected RBCs deformation lines). The deformation of infected RBCs [red line in Fig.6.7 (c)] was linear to the input flow rate. While the deformation of healthy RBCs [blue line in Fig.6.7 (c)] was very sensitive to the input flow rate, which immediately exceeded the half maximum while input flow rate was increasing and keep relatively large deformation (deformation>0.6,) most of the time (4 s out of 5s). This is similar with the healthy RBCs and low-concentrated artificially inelastic RBCs (0.01%) under the sine wave flow [blue and red lines in Fig.6.7 (a)]. The duty cycle is a helpful parameter to describe the wave profiles. Here we define the duty cycle in the following formulas:

$$\text{Duty cycle} = \frac{T_{\text{FWHM}}}{T_{\text{cycle}}}$$

where T_{cycle} is the total period of time for one cycle and T_{FWHM} is the total period of time for deformation larger than half maximum deformation. In figure 6.7 (d), the infected RBCs gave around 0.5 duty cycle (mean value 0.5553, standard deviation 0.05812, median 0.5423) which is the similar with one of a triangular wave while the healthy RBCs shows large duty cycle which is around 0.9 (mean value 0.8866, standard deviation 0.03459, median 0.8974). Same characteristic method was used for 0.01% tRBCs and healthy RBCs under sine wave flow (duty cycle is 0.6667). The results are indicated in figure 6.7 (b). The untreated healthy RBCs deformation gave around 0.9 duty cycle (mean value 0.8919, standard deviation 0.0603, median 0.9018) and the 0.01% tRBCs show around 0.7 duty cycle (mean value 0.6926, standard deviation 0.1471, median 0.7536).

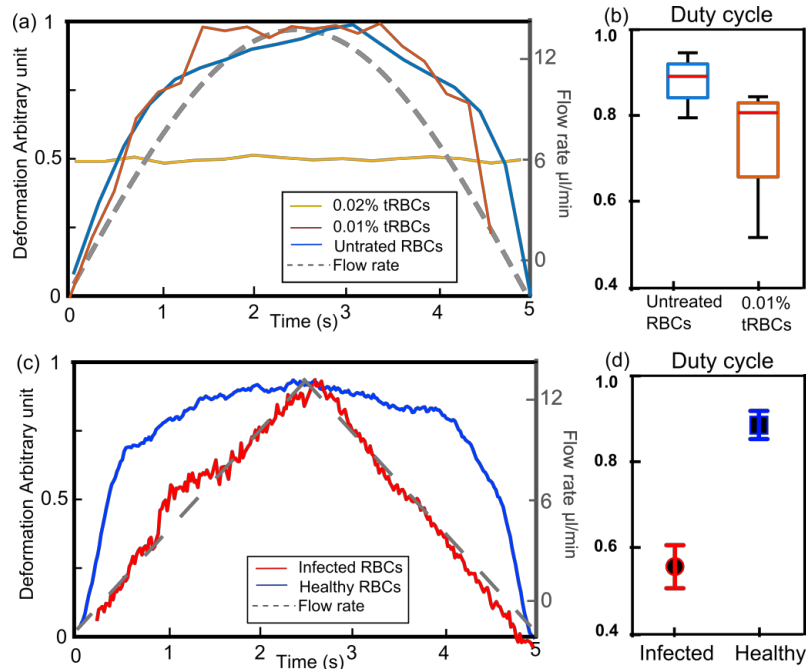


Figure 6.7 Deformation quantification for artificially inelastic RBCs, infected and healthy RBCs under the changing flow. (a) The yellow and red line represent the averaged deformations of 0.02%, 0.01% tRBCs respectively. The blue line represents the averaged deformations of healthy untreated RBCs. The grey dash line indicates the flow rate profile. (b) The duty cycle of deformation profiles in (a). (c) The red line and the blue line represent the averaged deformations of infected and uninfected RBCs. The deformation was quantified by the amount of shift (normalized) of centroid along the flow direction. The grey dash line indicates the flow rate profile. (d) The duty cycle of deformation profiles in (c).

6.4 Conclusion and discussion

In this chapter, a complete system and method have been developed for quantifying deformation of RBCs using DHM and microfluidic devices. The method required no sample staining and was capable of volumetric imaging/height mapping at video rate for multiple cells. The setting that RBCs adhered on the substrate allows the height map to be seen as 3D images. Since the bottom side of the cells was flat. And the cell stretching can be achieved by simply applying the hydrodynamic flow because of the tight adhesion. The deformability of the cells can be obtained through the analysis of the dynamic height maps. The trace of the centroid is one example of analysis methods and was adapted here to quantify the deformation. The analysis of the trace profile can reveal the deformability and the relationship between the deformation of the cells and the amount of force applied to the cells.

The study only contains a limited infected RBCs data which is not sufficient for any deformability conclusion from the infection. The main reason could be the livability of the infected RBCs under high shear conditions. The maximum flow rate (12.011 $\mu\text{l}/\text{min}$) of PBS solution (0.007 $\text{dyne}/\text{cm}^2 \cdot \text{s}$ viscosity at 37°C) with the minuscule channel size (100 μm width and 25 μm height) resulted in around 135 dyne/cm^2 shear stress, which only appears in stenosed arteries³⁶⁻³⁸, ignoring the microcirculatory blood flow where shear stress is irrelevant³⁶. The infected RBCs under this condition were vulnerable and lysed considerably. Therefore, reducing the maximum flow rate for infected RBCs and healthy RBCs is extremely needed.

There are other experiment possibilities that can be applied in this system for investigating the RBCs in other aspects. For example, the healthy RBCs showed rapid deformation when the shear stress occurs in the current experimental setting, while the infected RBCs deformation followed the flow increasing speed. One step forward to investigate this phenomenon detailed, the shear stresses that are increasing at different speeds could be tested to obtain the different speeds of RBCs centroids changing, which could provide the information about the reluctance of the cells.

In all, a functional DHM system with simple microfluidic method was proposed and developed for infected RBCs deformability study. More importantly, we have devised a double containment unit to operate under sterile conditions with DHM and microfluidics that will enable iRBC to be tested outside PC2 environment. The adhesion setting for RBCs in microfluidic makes fully use of DHM volumetric information and allowed the convenient hydrodynamic stretching implementation. The experimental setting still needs to be improved for infected RBCs and could be adapted to other investigation methods.

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Chapter 7 Thrombus dynamic studies under different shear rates using digital holographic microscopy

Publication relevant to this chapter:

He X, S J Montague, X T, E E Gardiner, W.M.Lee† “Quantifying embolism: label free volumetric mapping of thrombus structure and kinesis in a microfluidic system with optical holography” Advanced Biosystems, accepted (July, 2018)

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Contribution of others:

The work related to the sample preparation was done by S J Montague. The microfluidic chip fabrication was done by X T.

7.1 Background

7.1.1 From platelets to thrombus

Platelets are specialized anucleate blood cells that are essential for maintaining hemostasis, the process of maintaining a normal blood volume. A cardinal part of the process is the forming of a blood clot (thrombus). Thrombus formation is initiated by adhesion of circulating blood platelets onto exposed extracellular matrix proteins, including collagen and von Willebrand Factor (VWF), within a damaged vascular wall. Adhered platelets then release secondary agonists/mediators and promote thrombin generation, which helps recruit additional platelets to the injured site, cumulatively building a thrombus. Under pathophysiological conditions, this normally protective cascade of events can result in arterial thrombosis or deep vein thrombosis, which can be responsible for major clinical events such as heart attack and stroke.

Real-time monitoring of thrombus formation under fluidic settings is vital for studying the thrombotic process and provides significant insight to studying dynamic platelet function¹⁻². Studying the initial molecular events as a thrombus develops is crucial to understanding the involvement of key platelet receptors and can also help in assessing platelet dysfunction in patients with genetic defects². Measuring the growth of a thrombus would be useful in identifying targets to reduce pathophysiological events such as arterial thrombosis. Finally, the ability to study thrombus stability at pathological shear rates will inform on molecular

processes involved in embolization, and facilitate the development of therapeutic avenues to treat venous thrombosis.

A developing thrombus can be classified into a series of morphological transitions along a high fluid shear. The transition starts off with the initial activation of platelets through engagement of platelet signalling receptors which trigger activation of platelet integrin receptors and drive the aggregation of platelets that leads to thrombus growth before undergoing contraction ³⁻⁴. The contractile forces ⁵ from the platelet are in part due to the interaction with the surrounding fibrin network ⁶. On top of that, the degree of activation of each platelet in the thrombus influences how the platelets engage each other; fully activated platelets are held together within the thrombus by fibrinogen binding and bridging adjacent integrin receptors. Over time, adherent and fully activated platelets form the stable core of a thrombus, and newly-arrived, less-activated platelets are more loosely packed and held together forming a shell-like zone. The molecular composition of the thrombus core and shell affect the overall structural stability of the thrombus ⁷. To understand the basic biological processes underpinning thrombus growth and regulation of that growth, it is important to visualize the morphological development of a thrombus in real time using high speed imaging instruments that directly capture multiple views of a single thrombus and quantitates parameters including volume, height and area, simultaneously and temporally.

7.1.2 Imaging techniques for thrombus dynamics studies

Fluorescence intensity emitted from platelet-targeted fluorescent probes and surface markers are most commonly used to map out the overall architecture of thrombus ⁸⁻⁹. The two main fluorescence imaging methods are wide field fluorescence microscopy (WFM) and confocal laser scanning microscopy (CLSM) ¹⁰, which are introduced in chapter 2, section 2.2.2. In WFM, the changes of fluorescence intensity over a given area are used to estimate the volume of the thrombus. The advantage of WFM imaging is the speed of acquisition, which is limited by the frame rate of the imaging camera and also the brightness of the fluorescence signals. The fluorescence signal collected on a digital imaging camera is integrated from a single imaging focal depth of around 8 μm (based on

objective of magnification 10X and NA 0.25) ¹¹⁻¹³. This means that the capacity to measure the thickness of thrombus is restricted by the focal depth of the imaging lenses used. Therefore, if a thrombus is larger than the focal depth, the quantification would encounter inaccuracies in volume quantification ¹⁴⁻¹⁵. To circumvent this, most researchers have turned to more precise CLSM imaging that captures the whole thrombus through multiple 2D thin imaging slices recorded by raster scanning at a focused laser spot. This technique offers more accurate depth and thickness quantification along with area and density of a thrombus ^{2, 16-17}. The full 3D images acquired from CLSM imaging requires repeated scanning of a laser beam across the sample through mechanical devices such as galvanometer mirrors ¹⁸ of around 2 Hz at full frame or by spinning disc ¹⁹ that can reach 1000 Hz. The scanning process also exposes sample to higher light irradiation that can result in photobleaching ²⁰ or photodamage ²¹. In fluorescence imaging, there is an additional complication introduced by binding fluorescently-conjugated antibodies to the platelet surface to provide specificity. Recently, quantitative phase microscopy (QPM) techniques have provided new opportunities to measure thrombus formation *in vitro* ²²⁻²³. QPM maps out the difference in refractive index instead of using endogenous fluorescence signal and records thickness of the sample directly. However, since refractive index differences in biological samples are non-specific, this quantification tool requires a careful choice of biological samples ²³.

QPM is usually in two forms, non-holographic and holographic type. The non-holographic QPM ²⁴⁻²⁶ combines existing imaging optics of a WFM with numerical phase calculation and a prior knowledge to estimate the phase (refractive index difference) before it can generate a reconstructed volume of the sample. Although it has the same optical limitation as a conventional WFM, it provides volumetric quantification that is not available in conventional WFM, albeit at the low imaging speed. Holographic QPM ²⁷⁻²⁸, on the other hand, directly records the phase of the object and uses a non-focussed laser light. This method is not limited by the focal depth of the imaging optics in WFM or a focus laser beam in CLSM. The recorded phase also removes undesired optical distortion arising from any artefact or deformation within the sample ²⁹⁻³¹ which is important especially when dealing with samples of high scattering signals such as

blood samples. Recently, Boudejltia and colleagues³² showed that QPM can quantify individual platelet adhesion and other groups have also began to using non-holographic QPM techniques to quantify the mass of the final thrombus³³⁻³⁴. All in all, the application of QPM techniques in platelet and thrombus studies is in their infancy because of the lack of physiological relevant outcomes. We argue that QPM is uniquely positioned to present comprehensive and direct multi-dimensional morphological information of a developing thrombus, which would not be straightforward in other imaging modalities.

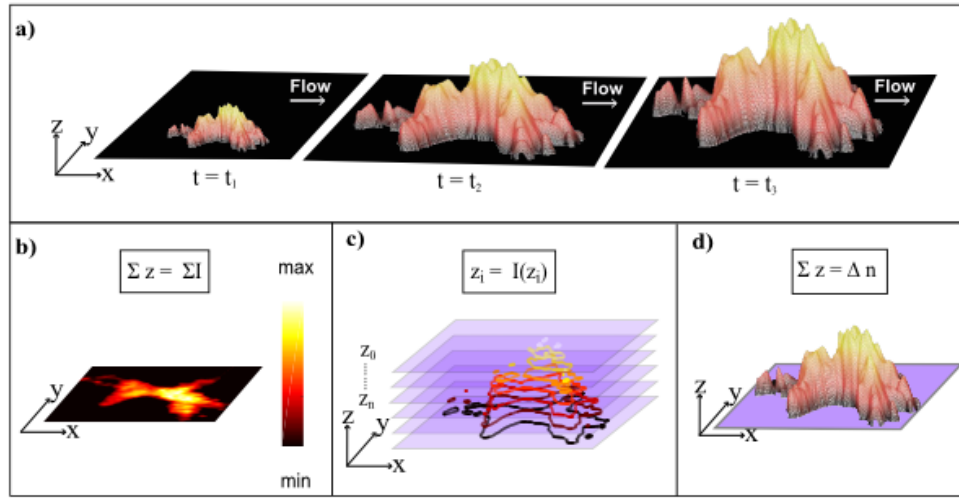


Figure 7.1 (a) Schematic diagram of a thrombus formed under flow. (b), (c) and (d) Comparison of three imaging techniques, Wide-field fluorescence microscope, Confocal laser scanning microscope and Digital holographic microscopy (DHM) respectively.

Table 7.1 and figure 7.1 present the three imaging modalities; WFM, CLSM and QPM that are typically used to quantify thrombus. Table 7.1 lists out the performances of the three modalities. Figure 7.1.a) illustrates the dynamic growth of a thrombus under flow at different time points (t_1 to t_3). Figure 6.1.b) shows how WFM imaging captures the integrated fluorescence over a given thrombus. Figure 6.1.c) illustrates the multiple thin image slices (Z_0 to Z_n) that depict recorded fluorescence intensities at each slice, $I(z_i)$, from which CLSM retrieves the thrombus volume. The repeated scanning of a highly focused laser beam causes an ineluctable lag time of the round-trip scanning which increases the overall acquisition time. Figure 6.1.d) shows how QPM imaging methods directly measure the refractive index difference of the thrombus which is directly translated to integrated volume. Another advantage in QPM is that the volume

does not depend on the brightness emitted from the sample or the dynamic range of the detector as compared to WFM. By demonstrating that QPM can measure dynamic events and parameters such as volume, height and area simultaneously and temporally at the different stages of thrombus formation and at multiple viewing points, this will enable a high throughput morphological mapping of thrombus development and greatly accelerate the understanding of early stages of thrombus development in a flow assay. A well developed and robust imaging framework using holographic QPM techniques also lends itself to on-chip holographic imaging techniques that be directly applied to diagnosing disorders of platelet function ³⁵.

Technique	WFM	CLSM	DHM
Molecular	Yes	Yes	No
Morphology	2D	2D and 3D	2D and 3D
Signal	Fluorescence brightness	Fluorescence brightness	Refractive index
Speed	0.0030 s camera frame rate	1 s	0.0030 s - 0.0006 s
Depth	0.4 to 5.8 μm (60x, NA 0.85 to 20x NA 0.4) ¹⁵	1 to 100 μm	> 0.1 μm *

Table 7.1 Summary of three imaging modalities; Wide-Field Microscopy, (WFM), Confocal laser scanner microscopy (CLSM) and Digital holographic microscopy (DHM). * Estimated based on 632 nm laser source with sample refractive index difference at 0.067.

7.2 Materials and methods

7.2.1 Microfluidic device

The microfluidic channel (400 μm wide, 100 μm height and 1.2 cm long) [Figure 7.2 a) ii)] was made of transparent polydimethylsiloxane (PDMS) by soft lithography method ³⁶⁻³⁷. Before the experiment, the entire microfluidic channel was coated with collagen Reagent HORM 100 $\mu\text{g/mL}$ (Takeda, Austria: a suspension of native equine tendon collagen type I) and incubated overnight at 4°C. The collagen was added through the outlet to ensure that platelets initially contacted collagen from the entry of the channel beyond the inlet. Before the start of the experiment, the channel was washed using a wash buffer (25 mM TRIS HCl, 150 mM NaCl, pH 7.4) perfused at 300 $\mu\text{L/min}$ for 2 min in the opposite direction of experimental flow.

7.2.2 Sample preparation

Venous whole blood was collected from consenting, healthy drug-free volunteers into syringes containing 3.2% trisodium citrate or 10 U/mL heparin (Sigma) as an anticoagulant. Ethical approval was granted by the Australian National University Human Research Ethics Committee (2016/317). Whole blood was supplemented with 40 μ M PPACK (D-Phenylalanyl-prolyl-arginine Chloromethyl Ketone; Cayman Chemicals) 5 min before experimental procedure.

Platelet-rich plasma (PRP) was prepared from anticoagulated whole blood by centrifugation at 200 g for 20 min and the upper PRP phase was collected. Prior to perfusion, blood samples were checked for absence of visible clots.

7.2.3 Image and data processing

The phase images were reconstructed from holograms recorded by CCD camera, using automatic numerical methods³⁸. The volumetric images were calculated from the phase images according to equations below using Matlab (MATLAB R2017a). Individual thrombus image (where $x, y \in T$) is selected from full field image by Otsu's threshold method³⁹. The thrombus characteristics evaluated were thrombus peak height, surface area and volume. The thrombus peak height H is the highest point across an individual thrombus image. The thrombus surface area S_{thrombus} is calculated from the summation of pixels covering the thrombus. The S_p is the actual size of one pixel represents. The value of S_p is calibrated through a 1951 USAF Glass Slide Resolution Target. The volume of thrombus V is calculated by multiplying the number of pixels that the thrombus covered by the volume of each pixel.

$$\text{height}(x, y) = -\frac{\text{Phase}(x, y) * \frac{1}{\lambda}}{2\pi * (n_{\text{pt}} - n_{\text{pa}})}$$

$$H = -\text{maximum}[\text{height}(x, y)], \text{ where } x, y \in T$$

$$S_{\text{thrombus}} = \sum_{x, y} S_p, \text{ where } x, y \in T$$

$$V = \sum_{x, y} S_p * \text{height}(x, y), \text{ where } x, y \in T$$

In the equations, x and y are the coordinates of the image, representing each pixel. Phase (x, y) is the phase image, and height (x, y) is the volumetric image. λ is the wavelength of the laser, which is 632.8 nm in our system. n_{pt} (=1.399) and n_{pa} (=1.332) are the refractive indexes of platelet and the buffer respectively 32, 40 – 41

7.3 Dynamic measurement

7.3.1 Thrombus formation

During thrombus formation, the different levels of activation of the platelets contribute to the overall packing density which affects physical stability of the adhering thrombus. During the initial stage of thrombus formation, there is a steady increase in volume which is followed by an overall thrombus consolidation/contraction ⁴². During physiological thrombus formation where fibrin is generated, the thrombus contraction is mediated in part by activated platelets pulling on fibrin networks to form a closely packed core ⁴³. The morphological change in the thrombus is orchestrated by mechanical and biochemical cues received by a formed thrombus. It has been showed that rate of diffusion of agonist within the thrombus is regulated by how platelets are packed ⁴². The anticoagulated blood used in this work to generate thrombi refers to the quantification of platelet deposition and platelet-platelet aggregation and that the process of fibrin generation was not studied.

To investigate the morphological changes in thrombus formation, we perfused PRP over collagen-coated microfluidic channels at three different flow rates that produce fluid shear rates that are physiological relevant to thrombus formation at 72 $\mu\text{L}/\text{min}$ (1800 s^{-1} shear rate; 18 dyne/cm^2 shear stress), 144 $\mu\text{L}/\text{min}$ (3600 s^{-1} shear rate; 36 dyne/cm^2 shear stress) and 288 $\mu\text{L}/\text{min}$ (7200 s^{-1} shear rate; 72 dyne/cm^2 shear stress) for 10 min. To investigate the parameters of thrombi formed under flow of whole blood, we perfused heparinized whole blood over collagen-coated microfluidic channels for 10 min at two different flow rates, with equivalent shear stress to PRP samples, 32 $\mu\text{L}/\text{min}$ (800 s^{-1} shear rate; 36 dyne/cm^2 shear stress) and 72 $\mu\text{L}/\text{min}$ (1600 s^{-1} shear rate; 72 dyne/cm^2 shear stress). The detailed conversion of shear stress, shear rate and flow rate can be found in Experimental section 7.2.3. The field of view for capturing dynamic

thrombus formation was fixed on sections close to the inlet within $\sim 800 \pm 200 \mu\text{m}$ after the inlet, for all the measurements to ensure consistency and minimal exposure to turbulent flow. Volumetric information was later reconstructed through classical holographic numerical approaches (see Experimental section 7.2.3). Figure 7.2 b) i) shows the result of the volumetric changes of thrombus formed at three different fluidic shear rates using PRP collected from heparinized blood from a single donor (d1). The x-coordinate indicates time in second and y-coordinate is for the volume in cubic micrometre. The blue, red and yellow lines indicate the thrombus formed under the 1800 s^{-1} , 3600 s^{-1} and 7200 s^{-1} shear rates respectively. Detailed statistic methods can be found in Experimental section 7.2.3. Figure 7.2 b) ii) shows the height map for each thrombus in figure 7.2 b) i) at different time points. The scale bar is $30 \mu\text{m}$ and the colour bar indicates the height in micrometres. The growth trends shown in figure 7.2 b) present a linear growth followed by inflection point, which is similar with previously reported growth trends^{42, 44} and mostly similar shape to growth trends of citrated PRP (Supplementary Figure A3.2). To quantify the growth rate, two linear fits (R-square above 0.9) were applied to the growth and inflection segments respectively, marked by black lines and S_1 and S_2 . S_1 represents the speed of the volume accumulation before the maximum point. S_2 represents the speed volume reduction after the maximum point. The statistics average of growth rate S_1 (the positive Y axis) and reduction rate S_2 (the negative Y axis) from all donors are presented in Figure 2 c) ii). The influence of the shear rate appears to be more dominant at 7200 s^{-1} compared with shear rates of 1800 s^{-1} and 3600 s^{-1} . While higher shear rates seem to suggest a rapid ascent, the speed (S_2) of the decline appears to be small ($< 1200 \mu\text{m}^3/\text{min}$) for all the thrombi formed at three different shear rates, which is likely to be related to platelet contraction³⁹ under flow. The quantitative observation of the events is made possible because of the robustness of holographic QPM sensitivity.

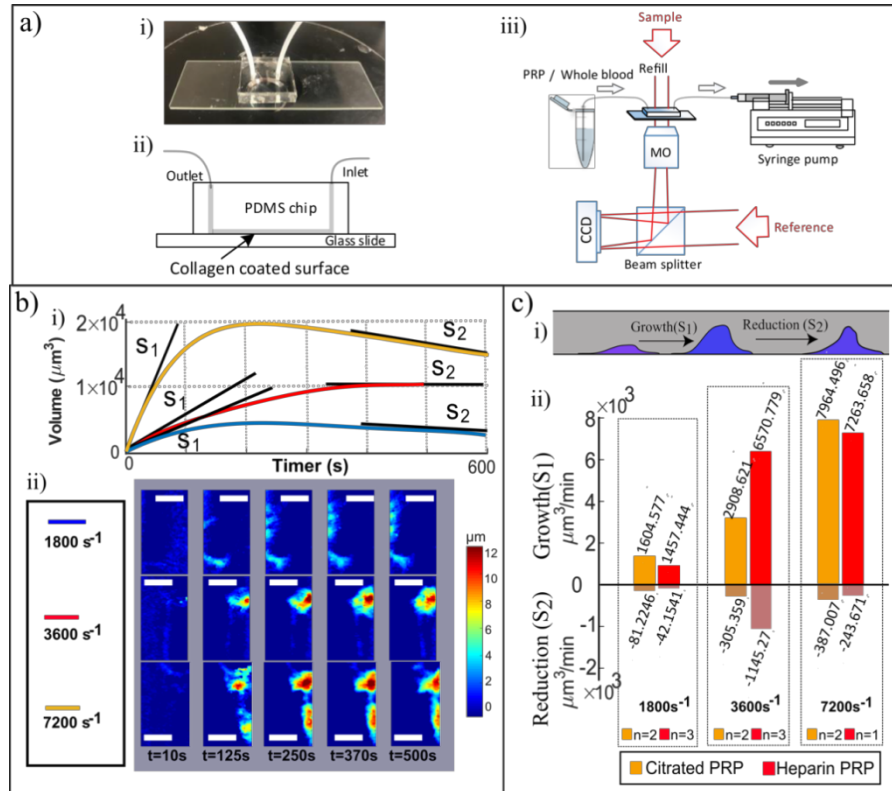


Figure 7.2 (a), Schematic diagram of experiment setup. (i), picture of polydimethylsiloxane (PDMS) chip used for experiment. (ii), schematic diagram of PDMS microfluidic chip setup. (iii) Platelet Rich Plasma (PRP) and whole blood (heparinized) as sample are perfused over collagen coated PDMS chips. Grey arrows represent direction of flow. MO is the microscopy objective. The red lines represent the laser light, and red arrows indicate the direction of laser light. Figure 2 (b-c). Thrombus formation result and analysis. (b) (i), Example volume growth of three thrombi formed by heparin PRP (from donor 1) under 1800 s^{-1} , 3600 s^{-1} and 7200 s^{-1} shear rates during 10 min perfusion of PRP. The lines in three colours represent three different shear rates. The lines are polynomial fits (with degree of 5 and R-square above 0.98) of smoothed (moving average filter) raw data. The black lines are the linear fit (R-square above 0.9) of the curve lines, representing the speed of the volume accumulations (the front part S1) and volume reduction due to contraction (the end part S2). (b) (ii) The height images show the thrombus forming at different time points. The scale bar is $30 \mu\text{m}$ and colour bar is in micron. Citrated PRP and whole blood thrombus volume growth curves are located in Appendix 3 [Figure A3.2]. (c) (i) schematic diagram of thrombus formation trend observed in the experiments. (c) (ii) The bar chart of average speed of volume change during thrombus formation. The positive Y axis is the speed of volume accumulation (S1). The negative Y axis is the speed of volume reduction (S2).

7.3.2 Thrombus stability

The spontaneous embolism of a thrombus especially in acute coronary events may be a natural protective mechanism against infarction in organs⁴⁵. Past studies have focused on individual platelet behaviour and more recently, there has been new interest in investigating factors related to thrombus stability that are able to be correlated with acute coronary events. Here, we investigate overall thrombus stability by perfusing a buffer at a high shear rate over a formed thrombus for a certain period of time and monitored thrombus parameters. The stability test could provide a new perspective to investigate the inner core and the outer shell regions of thrombus⁴⁶. Figure 7.3 a) illustrates the experiment undertaken where a thrombus that was previously formed with shear rates of either 1800 s⁻¹, 3600 s⁻¹ and 7200 s⁻¹ with PRP or at 800 s⁻¹, 1600 s⁻¹ with whole blood is then exposed to an additional saline buffer at a shear rate of 7500 s⁻¹ for 5 minutes and 12500 s⁻¹ for 2 minutes. To ensure a fair comparison of the volume, we selected aggregates with equivalent surface areas (1000 - 1800 µm²) and similar distance (~ 800 ±200 µm) from the channel inlet. Structural change during the flush could reflect the stability of the thrombus formed.

Figure 7.3 shows the stability test results. Figure 7.3 b) i) shows an example of the change in volume for three individual thrombi (pre-formed by 1800 s⁻¹, 3600 s⁻¹ and 7200 s⁻¹ shear rate, heparin PRP) from donor 4 (d4) during the stability test. Figure 7.3 c) i) shows the change in volume for thrombus pre-formed by 800 s⁻¹, 1600 s⁻¹ shear rate (equivalent shear stress as medium and high PRP shear rates) with heparinized whole blood from donor 4 during the stability test. The corresponding volumetric images are displayed in figure 7.3 b) ii) and 7.3 c) ii). Figure 7.3 b) iii), iv) and v) as well as figure c) iii), iv) and v) show the volume, surface area and height before and after the test. Each dot (circle for a thrombus formed under 1800 s⁻¹, triangle for 3600 s⁻¹, square for 7200 s⁻¹) in the bar represents the average data (obtained from n>8 data points) from each donor (D2, D3, D4 for citrated PRP, four donors for heparin PRP (d1-4) and four donors for heparinized whole blood). The statistical test performed was a paired t-test and significance was observed when p<0.05. There was decreased volume post-perfusion with citrate PRP at 3600 s⁻¹ (*p<0.05), due to a reduction in thrombus height (*p<0.05; Figure 7.3 b) iii and v). Reduction in height was also observed at

the same shear rate with heparin PRP, although there was no significant reduction in volume. The rapid decrease in volume with citrated PRP suggests an unstable outer thrombus layer that breaks apart with a residual core thrombus remaining in the same site. This leaves a thrombus of smaller volume and height. However, the surface area of the thrombus is maintained relatively, except for thrombi formed by heparin PRP at 3600 s^{-1} . In addition, the thrombus formed at lower shear rates (1800 s^{-1} and 3600 s^{-1}) retains a volume, area and height that are smaller than the thrombus formed at 7200 s^{-1} . We showed that holographic QPM can rapidly track, capture and quantify the rich dynamics of a developing thrombus.

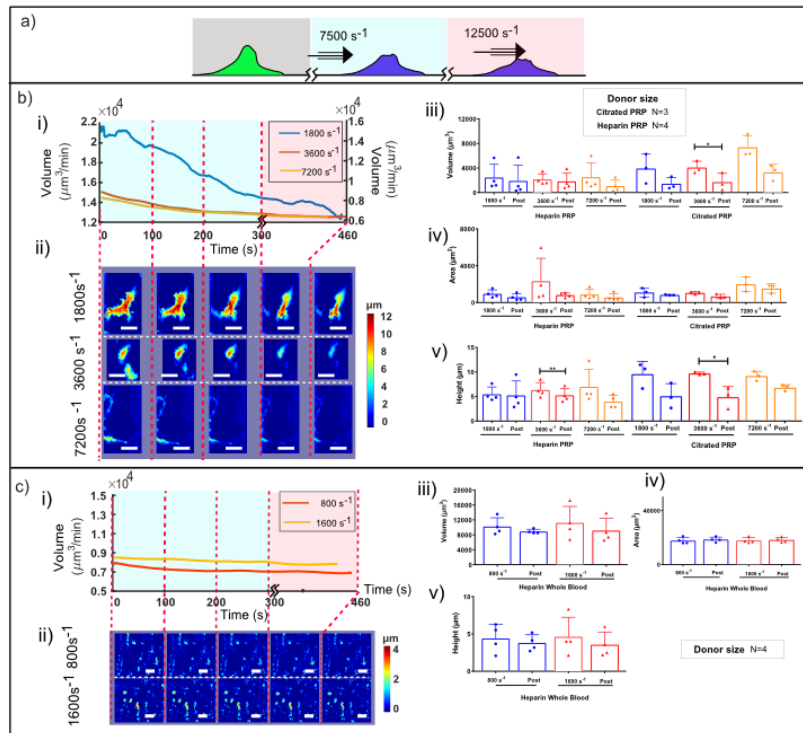


Figure 7.3 (a) Stability test by exerting high shear rate; buffer flowing at 7500 s^{-1} for 5 min and then 12500 s^{-1} for 2 min (b) Reduction of thrombus pre-formed by heparin PRP. i) Each line profile traces the thrombus volume that was previously formed under shear rate of 1800 s^{-1} (y-axis left), 3600 s^{-1} (y-axis right) and 7200 s^{-1} (y-axis right) from same donor (d4) respectively. ii) The images below the line plots are the corresponding thrombus at different time points. The scale bar is $40\text{ }\mu\text{m}$ and colour bar is in micron. (c iii-v) The bar graphs for average volume, surface area and the peak height data of the thrombus from all donors. Number of thrombus quantified, $n > 8$ from each donor. The statistical test is a paired t-test, $*p < 0.05$. Mean shown $\pm$ SD. (c) Reduction of thrombus pre-formed by heparin whole blood flow. i) Each line profile traces the thrombus volume that was previously formed under shear rate of 800 s^{-1} and 1600 s^{-1} from same donor (d4) respectively. ii) The images below the line plots are the corresponding thrombus at different time points.

The scale bar is 40 μm and colour bar is in micron. (iii) The statistics bar graph for averaged volume, surface area and the peak data of the thrombus from all donors. Number of thrombus quantified, $n > 8$ from each donor. The statistical test is a paired t-test. $*p < 0.05$.

7.4 Multi-parameter characterisation: volume, surface area, and peak height

After the 10 min formation process, we changed the flowing PRP or whole blood sample to the buffer (physiological saline; 0.9% NaCl) at the same shear rate previously used to remove excess PRP or whole blood and then translated the imaging field of view throughout the whole channel to capture images of individual thrombi. Using holographic QPM, we retrieved thrombus volume, surface area and maximum height after numerical reconstruction (see Experimental section 7.2.3) as shown in Figure 7.4. Figure 7.4 a) shows a perspective view of a reconstructed thrombus formed by PRP and whole blood. Figure 7.4 b) and c) show the measured volumes, surface areas and peak heights of thrombi formed by PRP and whole blood respectively. Each data point in the figure represents individual thrombi formed at different fluid shear (1800 s^{-1} , 3600 s^{-1} , 7200 s^{-1}). Differences were observed between thrombi generated with heparinized PRP and citrated PRP. The thrombi volumes appeared to be greater for citrated PRP thrombi that could be attributed to the increase in height without a significant increase in area of under which platelet is adhering to. Within the microfluidic system the thrombus height (heparin PRP and citrated PRP) ranged from $1.43\text{ }\mu\text{m}$ to $34.37\text{ }\mu\text{m}$ with median of $8.114\text{ }\mu\text{m} \pm \text{SD} = 4.933$. We observed that the volume and area of the individual thrombi from all the donors displayed significant degrees of variation. Hence, it would be of great interest to couple signatures between molecular and morphological information from each thrombus in future studies. However, this would require the combination of fluorescence imaging with holographic QPM⁴⁷ to give further information, which is explored in next chapter. By using the abundant characteristic information of individual thrombus morphology, it would be very informative to test functionality of platelets by controlled variables such as surface density of platelet receptors which control onset of platelet adhesion and thrombus formation. The phase images retrieved from digital holographic microscopy (DHM) provide the morphological information that is usually examined in platelet study using WFM

or CLSM at the same temporal resolution. In addition to that, the morphological information would help developed a better thrombotic numerical model ⁴⁵.

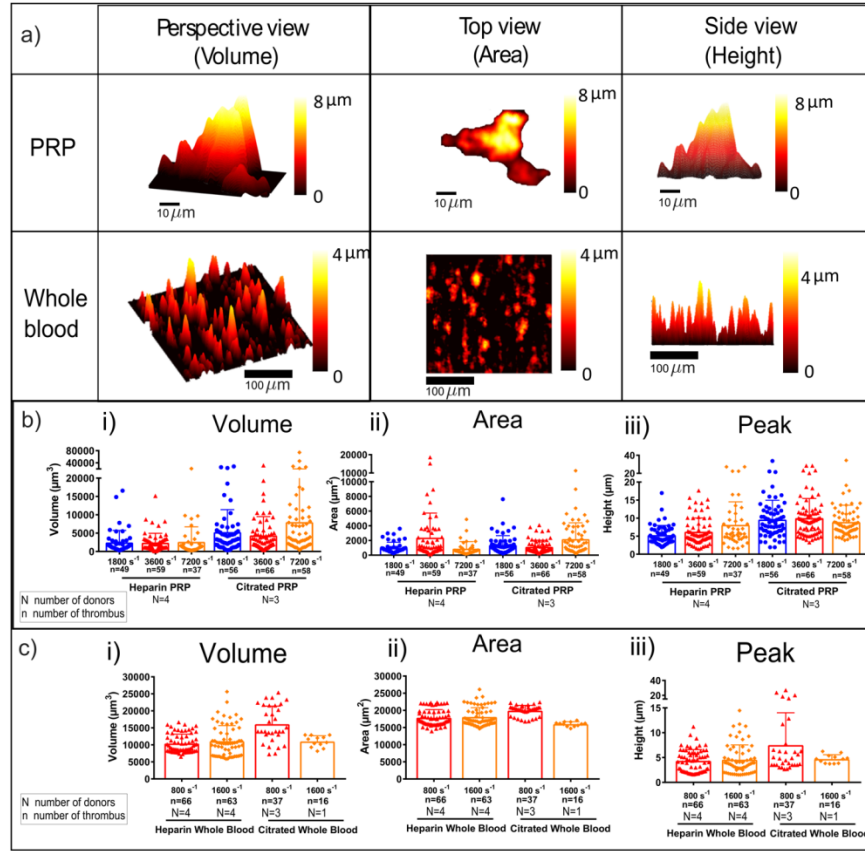


Figure 7.4 Individual thrombus characteristics. (a) Three columns show the perspective, top and side view of a thrombus formed by PRP and whole blood conditions. The side view is the cross sections of the thrombus through the peak point of it by the direction along with (Y axis) and the direction vertical to (X axis) the PRP and whole blood flow. (b-c) The volume, the surface area and the height of thrombus formed under different shear rates (PRP flow condition: 1800 s^{-1} , 3600 s^{-1} , 7200 s^{-1} ; whole blood flow condition: 800 s^{-1} , 1600 s^{-1} ; equivalent shear stress to mid and high PRP shear rates). The blue circle, red triangle and the yellow diamond are the individual thrombus formed under 1800 s^{-1} PRP flow, 3600 s^{-1} PRP flow (800 s^{-1} whole blood flow), 7200 s^{-1} PRP flow (800 s^{-1} whole blood flow). The number of thrombus is indicated by n beside each bar and the number of donors is indicated by N. Mean shown as +SD.

7.5 Conclusion

We have shown that the application of holographic QPM provides multi-parameter classification of morphological information in real time. Since depth of focus of the imaging system of QPM is not restricted, QPM retrieves the entire

morphological information set of a developing thrombus without any form of labelling. Without QPM, it would require considerable sample preparation and imaging time to retrieve the morphological information. Furthermore, whole blood presents a challenge in optical imaging as red blood cells (RBC) scatter light in a highly directional and ballistic manner, Mie scattering⁴⁸. By combining advanced digital signal processing techniques with digital optical holography this enables the removal of optical scattering and increases imaging resolution. With deterministic microfluidic parameters, the ballistic scattering property of RBC could be removed. We have previously shown that incremental thresholding allows one to retrieve images of an object embedded in a scattering medium³⁸ [chapter 5.4]. Further, Ferraro and colleagues have demonstrated the use of multi-frame holographic imaging to retrieve images of objects from flowing blood that are “hidden”⁴⁹⁻⁵⁰. However, imaging through turbid or scattered medium remains a major imaging challenge, with holographic microscopy being a key technique⁵¹⁻⁵². Many more recent techniques in optical holography⁵⁰⁻⁵³ can be applied to the analysis of thrombus formation in whole blood. However, one main limitation in the application of QPM is the non-specificity of the imaging technique that does not offer additional molecular information i.e. surface receptor levels. To achieve both morphological and molecular imaging, there is a need to combine QPM and high speed fluorescence imaging techniques such as light sheet microscopy⁵⁴. This provides a greater insight into the dynamic interaction of different receptors and adhesion molecules to thrombus formation. While high speed fluorescence microscopy requires specialized imaging sensors, holographic QPM can operate with more general imaging sensors. Hence, it is plausible to incorporate holographic QPM and microfluidic systems into a single portable unit. By reducing the footprint of holographic QPM and using unlabelled samples, it would be possible to use such a compact optofluidic system³⁵ to directly quantify dynamic thrombus morphology in a clinical setting. Here, we have used a limited number of healthy donors, which would need to be increased to permit comparative studies of thrombus growth rate with a wider distribution of shear rates. This will more accurately portray trends in volumetric growth and reduction over a wider distribution of shear rates. After which, from the clinical perspective,

such a system may be effective in testing anticoagulation therapy, identifying platelet dysfunction and monitoring pro-thrombotic stages.

7.6 References

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Chapter 8 Multimodality imaging system for microfluidic application

A multimodality imaging system, Holographic inverted Selective Plane Illumination Microscopy (H^+iSPIM), is proposed here. It is a hybrid system that combines DHM and an inverted light sheet microscopy. The purpose is to develop a simple hybrid imaging system, based on DHM and inverted light sheet microscopy, to provide both molecular and morphological images for biological cells in microfluidic devices. In previous chapters, a DHM system has been developed and employed [Chapter 5, 6, and 7]. In this chapter, different types of light sheet fluorescent microscopes, especially the inverted configuration, are introduced first. Next, the optical setup, the imaging reconstruction, and performances of H^+iSPIM system are demonstrated. The following part analyses the optical aberrations. At the end of the chapter, the discussion on improvements and future works of the system is provided.

8.1 Inverted light sheet fluorescence microscopy

Light Sheet Fluorescent Microscopy (LSFM), also called Selective Plane Illumination Microscopy (SPIM), provides two or three-dimensional fluorescence images through novel planar illumination, which was briefly introduced in chapter 1.2.1. Here, we shall discuss different configurations of LSFM in more detail, and problems associated with them such as uneven illumination issues. Figure 8.1 shows the diagram of the five main LSFM configurations in use today. The simplest configuration of LSFM [SPIM in Figure 8.1] uses one objective lens (or a cylindrical lens) to create a sheet of light to illuminate the sample from one side, and another identical objective lens perpendicular to the illumination sheet in the detection path. The excitation objective (or cylindrical lens) and the detection objective are placed around the specimen (in the same horizontal plane, x-y plane). However, this single-sided plane illumination configuration brings it with inherent uneven illumination due to the scattering and absorption of the illumination sheet as it propagates through the sample. The scattering and absorption result in stripes, shadows (ghost images), and intensity attenuation along the direction of the sheet throughout the images. These artifacts make it

difficult to accurately analyse the images. The next generation of LSFM uses both objectives (or cylindrical lenses) to produce the light sheet from each side and detect the fluorescence. Multidirectional Selective Plane Illumination Microscopy (mSPIM) ¹ and Multiview Selective-Plane Illumination Microscopy (MuVi-SPIM) have been developed to overcome the problems induced by single side illumination. The mSPIM uses two objectives and a scanning mechanism to produce a thinner pivoting sheet by scanning over a small angle at a high frequency (10° at 1 kHz ¹). The detection setting is the same as the initial SPIM described earlier. The pivoting of the light sheet removes ghost images in dense tissues and provides better contrast and better sectioning. However, the mSPIM also suffers from some drawbacks such as decreased time resolution and increased photobleaching and phototoxicity. The MuVi-SPIM applies two objectives for light sheet illumination and two objectives for detection to form four distinct illumination-detection pairs. This configuration yields a three-dimensional view without rotation of specimen or light sheet, and also solves the uneven illumination.

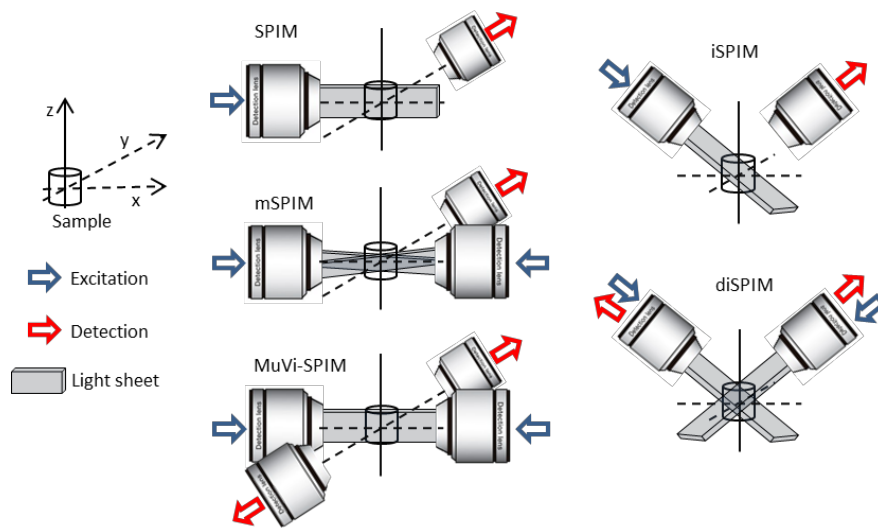


Figure 8.1 Different configurations of LSFM in three-dimensional space x, y and z.

Unfortunately, in SPIM, mSPIM and MuVi-SPIM configurations, the illumination and detection lenses are horizontally placed beside the sample. This usually results in requirements of unorthodox sample preparation, which often embeds the sample in an agarose gel (Figure 2.4.a)) ². Inverted Selective Plane Illumination Microscopy (iSPIM) ³ solves the sample preparation issue by

mounting two objectives symmetrically above the specimen (in the vertical z-x plane). Figure 8.1 shows the schematic diagram of the iSPIM. As a straightforward add-on to an inverted microscope, iSPIM allows conventional mounting of specimens, which leads to the prevalence of LSFM in biological studies ⁴⁻⁵. The impact of iSPIM on research areas such as three-dimensional cell studies, neuroscience and plant biology is increasing at a rapid pace. However, the issue of iSPIM is that if the sample was covered with a coverslip, which is a common requirement for biological samples, the fluorescent images would suffer undesired reflection from the coverslip. Dual/double-side inverted Selective Plane Illumination Microscopy (diSPIM) ⁶ was subsequently proposed. It makes use of the symmetry of the inverted configuration to implement illumination and detection on both objectives to solve the coverslip reflection issue as well as the uneven illumination of iSPIM. The diSPIM also provides better z resolution compared with iSPIM, but at the expense of imaging speed ⁶.

The iSPIM and diSPIM configurations are a straightforward add-on configuration for any inverted microscopy system. We shall make use of this advantage to design the H⁺iSPIM system and develop strategies for cells in microfluidic applications. The choice of iSPIM instead of diSPIM is considering the ability of fast imaging speed, which is vital for capturing dynamics in a fluidic flow.

8.2 H⁺iSPIM system: combine phase and fluorescence

8.2.1 Setup design

To build the H⁺iSPIM system, we installed a fibre based DHM on a commercial iSPIM (Single-side Inverted Selective Plane Illumination Microscopy, Applied Scientific Instrumentation). The iSPIM setup is complete original from Applied Scientific Instrumentation. The DHM setup is standard interferometer and similar with the figure 5.2 in chapter 5. The HeNe laser (632.8 nm, 5 mW, THORLABS) was used as light source for the DHM system and was coupled into a fibre splitter (50:50, 1x2 632 ± 15 nm FC/APC, THORLABS). One output was connected to a bare fibre (Single Mode, 600 - 770 nm, Ø125 µm, THORLABS) as the object arm and another output was connected to jacketed fibre of the same length (Single Mode Patch Cable, 633 - 780 nm, FC/APC, 1 m

Long, THORLABS) as reference arm. The reason for the bare fibre is that the two objective lenses (40X 0.8NA, 3.5 mm WD objectives Nikon) in the iSPIM configuration leave extremely limited space (between 0.2 and 0.5 mm) that would not accommodate a third microscope objective for DHM. Hence, a bare fibre with a diameter of 125 μm provides an ideal solution, which just fits between the two objective lenses and projects the laser light onto the sample. DHM, similar to bright-field microscopy, uses collimated light or divergent light but does not need focused light to illuminate the sample. That is also the key reason of using a bare fibre for H^+iSPIM system. An objective lens below the sample plane (white paper in the figure 8.2) collects the light passing through the sample, which is the object arm. The images acquired from DHM and iSPIM are from two different objectives, which requires matching the two field of views (FOVs). Hence, two translation stages were used to control the position of the fibre tip in the x and y directions. A single post clamp was used to hold the fibre tip, which can be manually adjusted along z direction. In the reference arm, laser light coming out from the fibre was collimated into free space and combined with the object arm at a sCMOS camera (Quantalux™, 2.1 Megapixel, THORLABS) by a beam splitter. Figure 8.2 provides the H^+iSPIM setup photos. The red laser light (632.8 nm) was projected from the fibre tip along the z direction to the sample plane (white paper in the photo) and the blue laser light (488 nm) was projected from the right objective lens at 45° to the z direction.

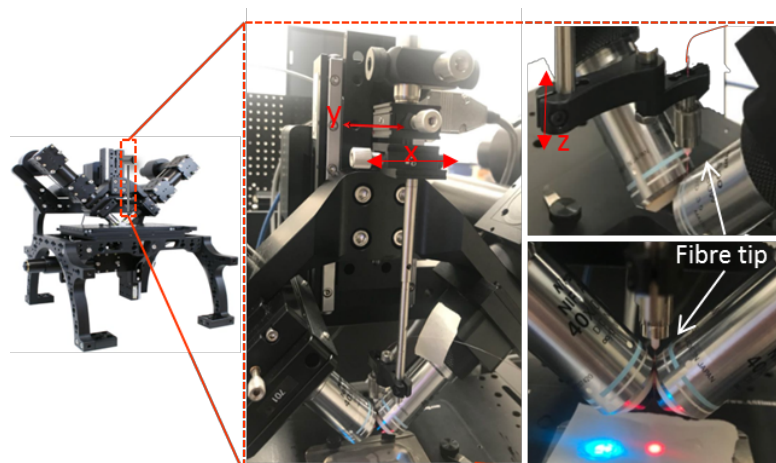


Figure 8.2 H^+iSPIM setup. The x, y and z indicate the three adjustment degrees of the fibre tip position. The red laser light is projected from the fibre tip along z direction to the sample plane (white paper in the photo) and the

blue laser light was projected from the right objective lens at 45° to the z direction.

8.2.2 Image processing and correlation

The imaging acquisition and processing of the two modalities are controlled independently. Therefore, additional signal synchronization in both spatial and temporal domains was needed to fuse DHM and iSPIM images for a single reconstruction. The imaging and reconstruction modes are demonstrated in figure 8.3. The imaging acquisition is described in the schematic diagram [Figure 8.3 a)]. The red laser is for DHM system and the blue laser is for iSPIM system. DHM captures images using camera 2 (Quantalux™, 2.1 Megapixel, THORLABS) and reconstructs two and a half dimensional (2.5D) morphological images. iSPIM illuminates the sample with MO1 (microscope objective 1) and fluorescent light is collected by MO2, relaying the 2D images to camera 1 (PCO sCMOS). The 3D images were reconstructed from a stack of 2D images at different depths.

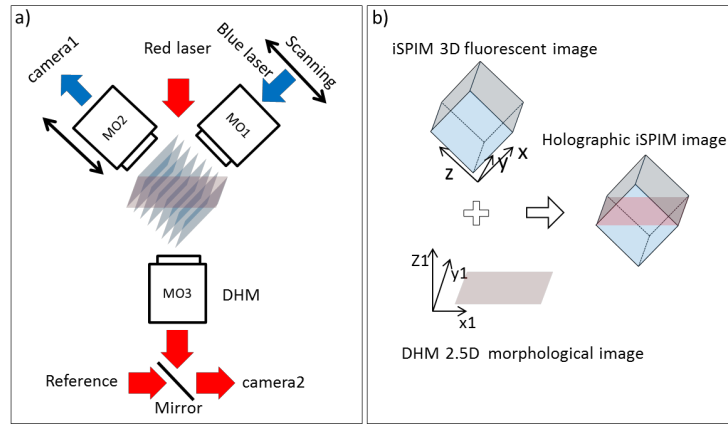


Figure 8.3 a) Schematic diagram of image acquisition. The red laser is for DHM system and the blue laser is for iSPIM system. iSPIM illuminates the sample with MO1 (microscopy objective 1) and fluorescent light is collected by MO2. 2D images were then captured by camera1. The 3D images were reconstructed by multiple 2D images in different depths. DHM captures images by camera2 and reconstructs two and half dimensional (2.5D) morphological images. b) Schematic diagram of image matching. The blue cube is the 3D image obtained by iSPIM and the red sheet represents the 2.5D image from DHM.

In order to match the 3D reconstruction (in x, y and z coordinates [Figure 8.3 (b)]) from iSPIM with the 2.5 D reconstruction (in x1, y1, and z1 coordinates Figure 8.3 (b)) from DHM, digital pixel reorganization of iSPIM 3D reconstruction is required to generate a stack of fluorescent images in the x1 y1 plane. Consequently, the number of scanning slices (N_s) determines the total number of pixels of newly generated iSPIM images in the x1 y1 plane. In this case, the scanning step distance d (μm) in z direction defines the pixel size of newly generated iSPIM images in x1 direction, which is equivalent to $\sqrt{2}d$. The scanning of two objectives is controlled by piezo objective movers (Applied Scientific Instrumentation f-POM-150), which provide a smallest step at 2.2 nm and a maximum travel range of 150 μm . The depth of field (DOF) is around 0.78 μm with 40X/0.8 objective and 488 nm laser, according to the equation: $\text{DOF} = \lambda / \text{NA}^2$. To reach the optical limit axial resolution (z axis), sampling/scanning interval is required to be smaller than a half of DOF to satisfy the Nyquist–Shannon sampling theorem, which requires the scanning step distance d to be smaller than 0.39 μm . Therefore, we shall define the resolution in the x1, z1 plane as R_{x1z1} . The value of it is 1.1 μm ($\sqrt{2}$ DOF) when scanning step distance d is set smaller than 0.39 μm . The R_{x1z1} at $2\sqrt{2}d$ (length of two pixels) can be achieved when the scanning step distance d is set larger than 0.39 μm . The pixel size of newly generated images in the y1 direction remains the same as the original obtained images in the x-y plane, which is defined by width of FOV (471 μm) and pixel number of camera 1 (2048*2068). The resolution in the y1 direction is not affected by the scanning setting and complies with the equation: $0.61 \lambda / \text{NA}$ (0.37 μm), which is defined as R_{y1} . The image speed can be estimated by the number of scanning slices N_s , time for taking one scan (20 ms) and the scan settle time (3 ms). The detailed list and calculation methods are listed below table 8.1.

iSPIM Parameters	Symbol	Type	value
Scanning step distance	d	Input	2.2nm
Number of scanning slices	N_s	Input	Maximum 150 μm
Scanning depth	$N_s * d$	Variable	
Width of light sheet	W	Fixed	471 μm
Length of light sheet	L	Fixed	474 μm

FOV (x y plane)	W*L	Fixed	471*474 μm
FOV x1y1	$N_s * \sqrt{2}d * W$	Variable	Maximum 212 *471 μm
R_{x1z1}	1.1 μm ($d < 0.39 \mu\text{m}$) $2\sqrt{2}d$ ($d > 0.39 \mu\text{m}$)	Variable	Minimum 1.1 μm
R_{y1}	0.61 μm /NA	Fixed	0.37 μm
Acquisition time	$20 * N_s + 3$	Variable	Minimum 23ms

Table 8.1. iSPIM setting and performance list

Therefore, in this modality, the imaging performance of the iSPIM is according to the relationships listed in table 8.1. The resolution of the 3D reconstruction in x1 and z1 axis, the image speed, and the FOV all need to be carefully weighed and chosen according to different applications. In biological studies, the spatial and temporal scale of the observation window usually has a fixed range, which determines the requirements of FOV and the image speed of the system. Therefore, we shall explore four setting modes for different FOV and image speed. Figure 8.4 represents two FOV choices. In figure 8.4 a), maximum scanning depth of 150 μm is applied to reach the largest FOV (212*471 μm). Based on this FOV, mode 1 with 142 scanning slices at 1.06 μm step size is aimed to reach relatively rapid acquisition time (2.8s) and rough resolution (1.5 μm /pixel, 3 μm resolution in x1 z1 axis). Mode 2 is for maximum resolution with maximum FOV. Similarly, figure 8.4 b) provides the smaller FOV (50*471 μm) with fast image (mode 3) and maximum resolution (mode 4). The specific performance of each mode can be referred to in table 8.2.

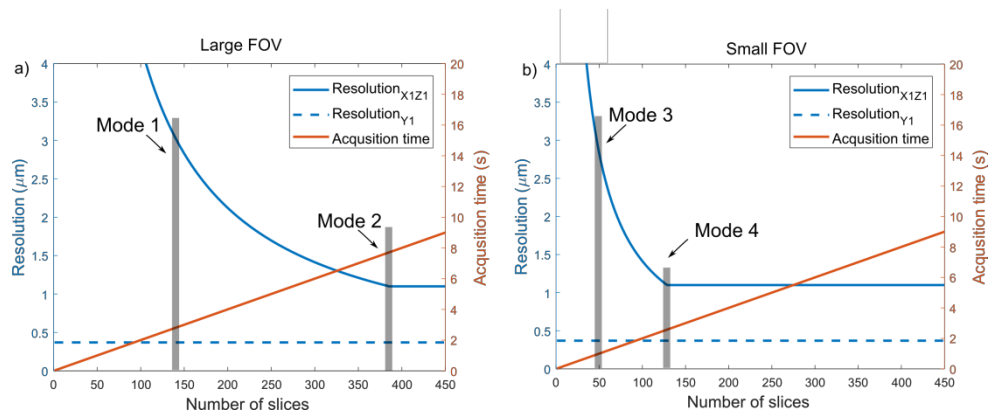


Figure 8.4 Performances of iSPIM in different modes. The relationship among 3D reconstruction image resolution, acquisition time and the number of scanning slices for a) Large FOV of 212*471 μm . b) Small FOV of 50*471 μm . Four operation modes are indicated by grey lines and arrows with text.

iSPIM Parameters	Type	Mode 1	Mode 2	Mode 3	Mode 4
Scanning step distance	Input	1.06 μm	0.39 μm	1.11 μm	0.39 μm
Number of scanning slices	Input	142	385	48	129
Scanning depth	performance	150 μm	150 μm	50 μm	50 μm
FOV $\times$ l y l	performance	212*471 μm	212*471 μm	71*471 μm	71*471 μm
Resolution $_{X1Z1}$	performance	3 μm	1.1 μm	3 μm	1.1 μm
Resolution $_{Y1}$	performance	0.37 μm	0.37 μm	0.37 μm	0.37 μm
Acquisition time	performance	2.8s	7.7s	0.96s	2.58s

Table 8.2 iSPIM setting and four performance modes

8.3 Strategy for microfluidic study by H⁺iSPIM system

8.3.1 Light sheet microscopy for biology study in microfluidics

The novel illumination technique of LSFM offers it the abilities of less photobleaching, faster imaging speed, longer specimen life, and mapping entire organism with single cell resolution. Thus, the LSFM family has been rapidly developed and applied to a wide range of applications. However, it is also the novel illumination that brings about the arduous process of sample mounting and preparation⁸. For example, in iSPIM system, the cover glass which is commonly used for conventional microscopic sample needs to be avoided due to the introduced aberration when imaging through 45 degrees. In microfluidic experiments, which are increasingly employed in biological research, the flat cuboid chips with microchannels inside are the most common devices for the samples and liquid. These chips are appropriate for microscopes where the illumination and detection are collinear, but they are awkward for both SPIM and iSPIM where illumination and detection are orthogonal. However, a coverslip or other sample mounting/container, for example a microchannel, is inevitable for some of biological experiments where the sample needs to be protected or supported in a special environment. As a result, different strategies and devices have been proposed. Figure 8.5 represents some examples of them. McGorty *et al.*⁹ have employed a water prism between the cover glass and objectives to compensate for the aberration induced from the flat cover glass. Miura *et al.*¹⁰ developed a customized mirror-embedded microfluidic chip which enabled light sheet excitation and detection with one objective [Figure 8.5 b)]. Deschout *et al.*

designed a microfluidic chip with an integrated planar waveguide for light sheet illumination [Figure 8.5 c)]¹¹.

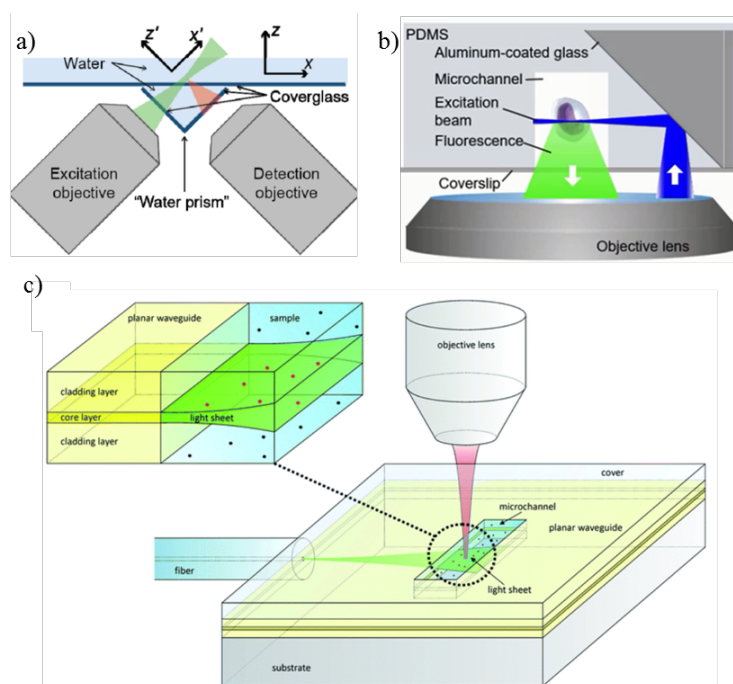


Figure 8.5 Strategies for LSFM microfluidic applications. a) A water prism based sample amount design from Ref. ⁹, reproduced with permission from © 2015 Optical Society of America. b) A customized mirror-embedded microfluidic chip developed from Miura et al. ¹⁰. Picture is reprinted with permission from © 2018 Optical Society of America. c) A microfluidic chip design with integrated planar waveguide for light sheet illumination, from Ref. ¹¹, reproduced with permission from The Royal Society of Chemistry.

8.3.2 Aberration from a flat cover

All the configurations introduced in figure 8.5 were designed to avoid directly illuminating the sample and detecting the fluorescence signal at a 45° angle with a flat surface (cover glass). In order to develop microfluidic strategies for the H⁺iSPIM system, the aberration induced by a flat cover at 45° angle is analysed in this section. Standard fluorescent microspheres (Fluoresbrite® YG polystyrene Microspheres, Calibration Grade 3.00µm) were imaged by iSPIM system. Figure 8.6 a) demonstrates the imaging result of a sphere without the coverslip on top, while b) demonstrates the result of a sphere below a standard coverslip (22*50 mm, #1 MENZEL-GLASER). The purple spheres are the theoretical 3D shape of the spheres, and the yellow contours are the actual 3D reconstruction of the iSPIM fluorescent images (0.1 µm scan step size, total 100

steps). The thumbnails below the reconstructions are the iSPIM fluorescent images at different depths with an interval of 1 μm (10 slices). The scale bar is 5 μm for all images. The 3D contours were reconstructed based on the half maximum intensity of the fluorescent images. The result of the sphere covered by a coverslip showed highly distorted images and reconstructions, which presented a tail-like structure. We conclude that the coverslip close to the sample introduces extremely strong reflection of fluorescent light and significant aberrations.

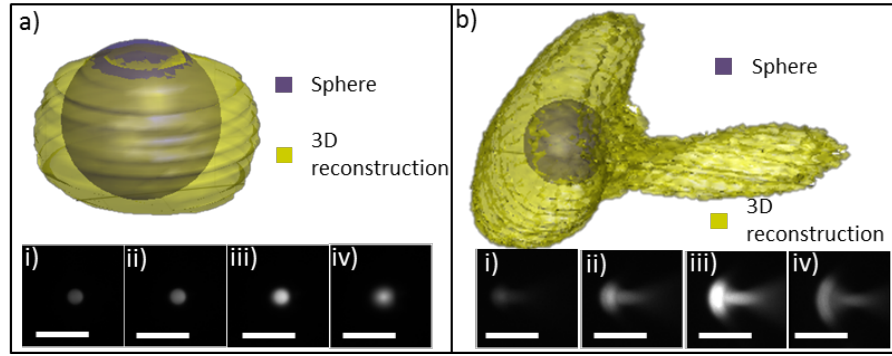


Figure 8.6 iSPIM images and reconstructions of microspheres without and with a coverslip. The purple spheres are the theoretical 3D shape of the spheres, and the yellow contours are the actual 3D reconstruction of the iSPIM fluorescent images indicated by i), ii), iii), and iv). The scaler bar is 5 μm for all images. a) Result of a 3 μm sphere. b) Result of a sphere below a standard coverslip.

8.3.3 Aberration from a round microfluidic channel

The next step towards microfluidic strategies for the H^+ iSPIM system is to evaluate the aberration from the common round tube, which is the most simple and symmetrical microchannel that can be used in microfluidic applications. The same microspheres were used in a 1 mm diameter round tube (capillary tube 1.0 mm OD, 0.01 mm thickness, Hilgenberg GmbH) filled with gelatine for fixation. Figure 8.7 a) shows the side view of the positions of the tube and objectives. The black circle indicates the cross section view of the tube. The zoom-in schematic diagram is shown in figure 8.7 a) ii). The bright-field image of the tube is presented in figure 8.7) iii). Figure 8.7 b) and c) demonstrate the reconstruction of microspheres in two positions within the tube, marked by the blue and red arrows. Below that the purple spheres are the theoretical 3D shape of the spheres, and the yellow contours are the actual 3D reconstruction of the iSPIM fluorescent images

(0.1 μm scan step size, total 100 steps). Figure 8.7 b) is the result of a sphere inside the tube. The shortest distance between the tube wall and the sphere was 60 μm . The intensity images showed no reflection effect and only minor distortions from round to ellipse. For the spheres located deep inside the tube (more than 60 μm distance way the tube wall), the distortion of the 3D reconstruction become smaller. Figure 8.7 c) shows the result of a sphere close to the tube wall (the distance was smaller than 10 μm). The reflection effect was observed in intensity images at certain depths. However, the reflected intensity was weak and smaller than half of the maximum intensity of the spheres. In this case the 3D reconstruction showed no tail-like structure that was seen in figure 8.6 b). Nonetheless, the 3D reconstruction was distorted to a slightly greater extent compared with the 3D image of the sphere inside the tube.

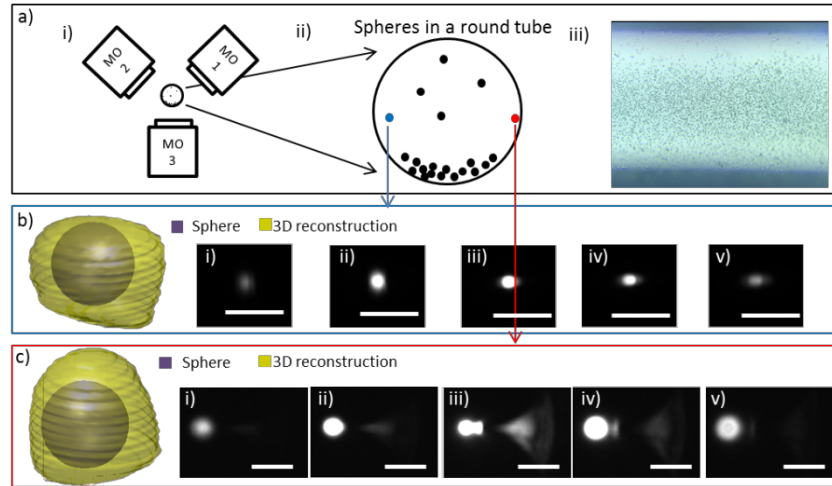


Figure 8.7 iSPIM images and reconstructions of microspheres in a round tube. a) i) schematic diagram of orientation of sample and objectives, ii) the enlarged cross-section view of spheres in round tube and iii) the bright field image of spheres in the tube. b) The image result of a sphere inside the tube. c) The image result of a sphere beside the tube wall. The purple spheres are the theoretical 3D shape of the spheres, and the yellow contours are the actual 3D reconstruction of the iSPIM fluorescent images indicated by i), ii), iii), and iv). The scaler bar is 10 μm for all images.

8.4 Conclusion and future works: phase and intensity

The same microsphere was imaged with the DHM system. The phase profile of the microsphere is indicated in figure 8.8 (a) with a top view and a perspective view (scale bar is 2 μm). Figure 8.8 (b) represents the overlap of the 3D reconstruction of light sheet image, a theoretical 3D profile of the spheres

[Figure 8.6 a], and the height map from DHM. From figures, it is clear that the combination of images from DHM and light sheet is not a straightforward process for three-dimensional objects. The phase profile from DHM reveals the total optical thickness along the optical axis (vertical in this case). However, it does not reveal the distribution of the thickness vertically. The images from iSPIM render the distribution of the specimen volume by scanning spatially. Therefore, the H^+ iSPIM system can be used for adhesion assay (chapter 6) and thrombus dynamics (chapter 7), in which the bottom side of the subjects are assumed flat. However, for other situations, the phase profile cannot be regarded as the 3D structure of the samples. A promising utilization of the phase profile in H^+ iSPIM system is to map the refractive index within the sample. The actual thickness of the sample can be obtained with iSPIM system. Combined with the optical thickness map from DHM, the refractive index map of the sample can be easily calculated. Also the local refractive index change can be acquired over time, which is valuable in investigating the nuclear refractive index changes during the cell cycle or during canceration¹²⁻¹³.

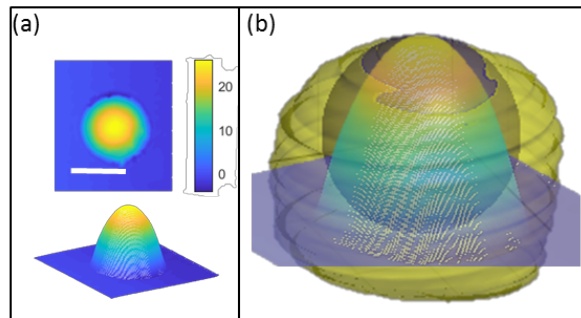


Figure 8.8 (a) Microsphere was imaged with DHM system. scalar bar is 2 μm .
(b) overlap of 3D reconstruction of light sheet image, theoretical 3D profile of the spheres [figure 8.6 a], and the height map from DHM.

In conclusion a multimodality imaging system, H^+ iSPIM, is proposed for both molecular specificity and morphological reconstruction. The image combination and aberration evaluation were discussed. In the future work (next chapter), microfluidic strategies are proposed for the H^+ iSPIM system with the aim of simple adhesive assay applications. In addition, the improvement of the H^+ iSPIM system in the optical part can be another future direction. DHM can provide both phase and amplitude of the light that diffracted by the sample at any plane along the z axis by digitally propagating the wave front (explained in

chapter 5.3.3). The obtained phase information also contains the information from the sample mounting, for example the coverslip or microchannel. Hence this phase information potentially provides the key for removing the aberrations primarily introduced by sample mountings.

8.5 References

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Chapter 9 Conclusion and future works

9.1 Conclusion of the thesis

Due to the rapid development of biomedical imaging instruments, it is essential for imaging techniques to possess high-resolution images, easy accessibility for non-technicians, and adaptability for a wide range of applications. On this basis, the groundwork of the thesis is to automate the DHM system to make it user-friendly and flexible for different applications and scattering environments. The following work is focused on applying microfluidic flow assays to studies of RBCs and platelet biomechanics. Moreover, a novel multimodality system called H⁺iSPIM was proposed as a combination of DHM with another promising and novel fluorescence imaging modality, light sheet microscopy. As a result, multifunctional images can be provided by the hybrid system, which is potential to achieve comprehensive monitoring and measurement of biological events. The microfluidic strategies for aberration-free imaging are proposed in the next section as a future work. Overall, the thesis focused on developing advanced volumetric microscopy techniques for dynamic studies of cell mechanics using simple and efficient microfluidic assays.

In the following conclusion sections, the limitations of the thesis work are discussed. Ideas of how to overcome these limitations and improve the system are provided as future works.

9.2 Portable holographic system for on-chip diagnosis by mobile phones

As mentioned in chapter 4.3.1, the simple, low-cost microfluidic chip is a great choice for on-site diagnosis and point of care services which is in great

demand. Currently, most on-chip diagnosis methods make use of macroscopic indicators like colour changes of the reagent by chemical tests (regularly used in virus detections) or timing the blockage process of the channels (commonly used in blood clot tests). The on-site cellular analysis of the dynamic events in microchannels is still uncommon, most likely because it is inconvenient and expensive to implement laboratory microscopy with the microfluidic chips. Fortunately, this situation is expected to change rapidly with the recent technological developments and system miniaturization on QPM modalities. A variety of QPM miniaturization approaches have been developed recently¹⁻⁵ to achieve compact, low-cost, alignment-less or even lens-less imaging solutions. Some of these provided a small extension to inverted microscopy^{1,5}, while some shrunk the entire microscope into a small device that can fit into a pocket²⁻³. Apart from system miniaturization, the simple image processing and statistical analysis is another important add-on for remote medical diagnosis and point of care tests. From this point of view, smartphone based portable microscopy has come into being and gradually become popular⁶⁻¹⁰. For example, Figure 9.1 shows a hand-held smartphone based QPM device developed by Meng *et al.*⁷, which is a compact microscope system with a cost-effective illumination source. The only drawback is that it retrieves phase information from multi-plane intensities. Therefore, it is more suitable for static sample inspection.

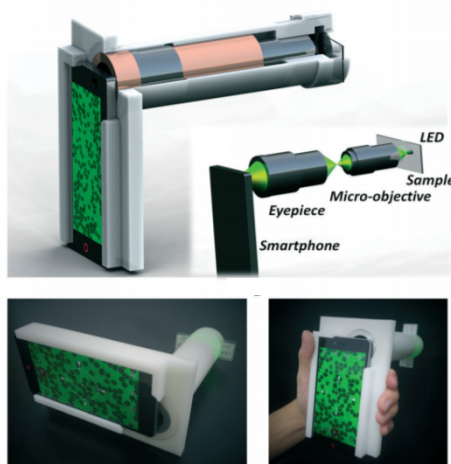


Figure 9.1 Portable microscopy techniques. Reprinted from reprinted from Ref. [7] with permission from The Royal Society of Chemistry

Based on these promising technique developments, we shall draw the blueprint of a portable holographic system for handy on-chip diagnosis by mobile

phones. Shown in figure 9.2, the conventional off-axis interferometer configuration can be retained. This maintains the single image reconstruction ability, thereby allowing imaging of dynamic events in the chips. The system is designed to be cost effective, increasing its accessibility in remote diagnosis applications. It has potential to be used as on-site blood test for situation outside hospital. The system consists of a low-cost laser pointer as the light source, two plate beam splitters, and two mirrors as the basis of the interferometer, which are standard mass-produced optical components of relatively small size. In addition, PDMS microlenses¹¹⁻¹² can be used instead of a conventional optical lens, which further reduces the cost. An adjustable clamp is required to align and fix the device on different smartphones. The frame along with the clamp can be 3D printed¹³, which allows for flexible and rapid prototyping at low cost. However, the deforming and bending of the frame material could impair alignment significantly. Therefore, the material and the printing techniques should be chosen carefully. The pump system of the diagnostic microfluidic chip can be designed in a capillary force driven configuration. The principle and the importance of capillary forces in microfluidics have been introduced in chapter 4.1.3, which are related to surface tension driven fluidic systems. More detailed principles and applications can be referred to in references¹⁴⁻¹⁶. The overall system is a portable device around 50 cm³ in size. All of the image processing and quantitative measurements can be performed by smartphones. A primary diagnosis can be obtained by the proposed design within a short time and with low cost.

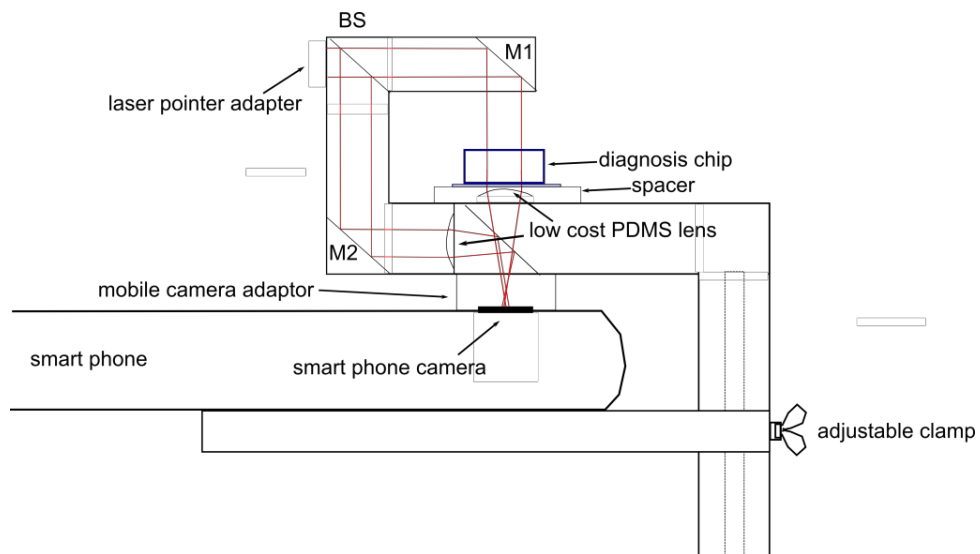


Figure 9.2 A design of portable holographic microscopy for handy on-chip diagnosis by mobile phones.

9.3 Challenges in imaging through scattering fluid

Another future research direction of the thesis is targeted at optics, specifically the ability of imaging through scattering fluid, as in blood flow. Imaging through blood flow is demanding in numerous biological studies both *in vivo* and *in vitro* (on chips). For example, the platelet aggregation study in chapter 7 could reveal highly valued physiological information if dynamic aggregation of platelets can be directly recorded and measured under whole blood perfusion. This would require an imaging technique with excellent ability to image through turbid or scattering media. In chapter 5.4, the ability to image microspheres under a scattering hydrogel medium has been demonstrated. However, that was in a low scattering situation. Unfortunately, blood is an extremely scattering case due to the density of RBCs and their considerable size. A one-microliter drop of blood has more than 4 million scattering particles, RBCs, which are individually around 6 μm in diameter. This size is very large, especially when compared to traditional biological samples of interest in blood (platelet aggregation in an early stage with the dimension of less than 20 μm diameter for example). Unlike inverted fluorescent microscope that images the samples beneath a blood flow from the opposite side, most non-labelled imaging techniques for biological study use the transmission of light through the sample and the surrounding medium. Therefore, imaging through blood flow has been a big challenge in the field of non-labelled imaging techniques for a long time. There have been a lot of efforts made to develop different approaches to retrieve clear images through scattering media using various coherent imaging techniques¹⁷⁻¹⁹. For example, Pietro Ferraro's group has shown promising DHM images of static objects under flowing blood²⁰ by taking multiple frames of holograms. For all the methods, additional frames of images or sample displacements are required to reconstruct a clear image. To adapt those methods to our applications, two things need to be considered. First, there is always a trade-off between imaging speed and image resolution. This situation is analogous to laser scanning microscopy when capturing a volume of a sample during a dynamic event. Secondly, a dynamic adjustable strategy is required to cover the whole platelet aggregation process since the size of the

aggregation is constantly changing. The volume of the aggregation will increase from zero to a bulk (hundreds of microns). This is much bigger than the RBCs responsible for the scattering. The ratio of target diameter (from zero to hundreds of microns) to scattered particle diameter (6 μm) is crucial for choosing and optimising the image retrieval algorithms.

In conclusion, the DHM system inherently has the ability to image thrombus formation in whole blood. However, more groundwork is needed to develop a robust and tailored image retrieval method.

9.4 Modelling cells in microfluidics for biomechanical measurement

The third future work of the thesis is aimed at the further utilization of dynamic morphological information from DHM system. For example, the RBC deformability study in chapter 6 explored morphological changes by tracking the centroid of the cell. The method is valid and simple but lacks panoramic views of the RBCs. The height map provided by the DHM system pictures the entire cell. One way to make full use of this information is to build the model of the investigated cell with computational methods based on the experimental data, and obtain the specific physical response quantitatively. As introduced in chapter 3.2, RBCs consist of mainly three components, the fluid cytoplasm (haemoglobin), the lipid membrane and the membrane skeleton. The physical properties of the three have been comprehensively studied for decades. Various computational modelling methods for RBCs have been proposed and successfully applied to cell biomechanics studies²¹⁻²³. It is highly promising that the mathematical modelling of RBC based on the height map from DHM images can render better investigation of an entire RBC response both spatially and temporally. Figure 9.3 shows an initial step of the mathematical modelling of RBCs under increasing shear stress. The work was done in collaboration with Dr Vijay Rajagopal and Dr Maciej Kubicki from the Cell Structure and Mechanobiology Group in the University of Melbourne. The initial biconcave shape is obtained by the formula of Evans and Fung²⁴. The arrows represent fluid flow around the RBC. The continuum mechanics, finite element modelling approach, was used to simulate the mechanical deformation based on the single layer hyperelastic model²⁵⁻²⁸.

The model can be a basis for further development and refinement by experimental observations. In the next step, the experimental height map will be added to generate the initial shape of the cell, and the experimental shear stress will be input based on the physical flow condition. This will allow the forces that were acting on the RBC to be accurately modelled and calculated. Thus, the mechanical stiffness of the cell can be estimated.

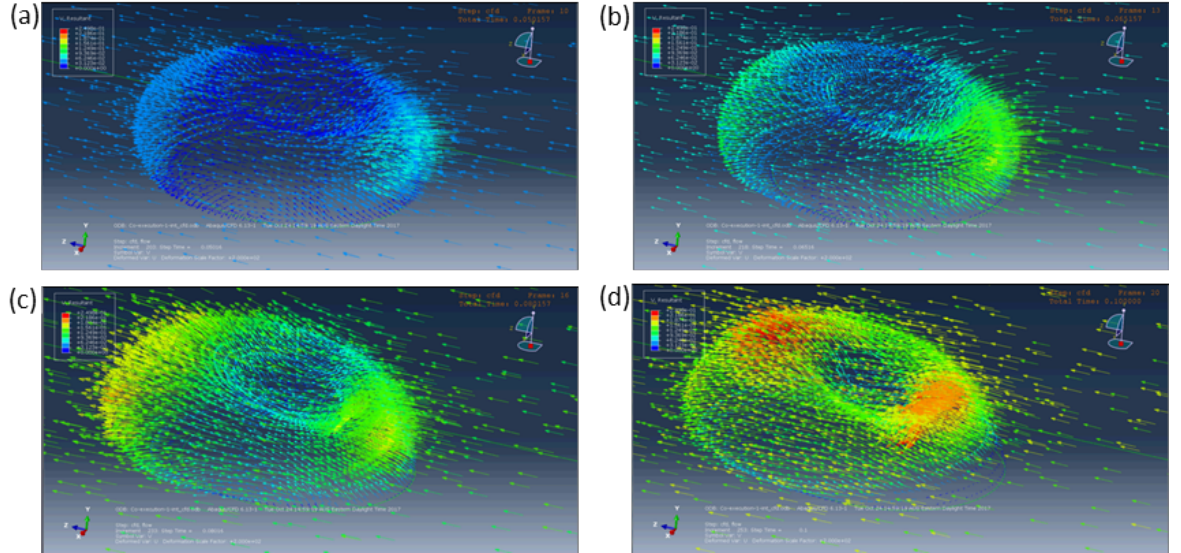


Figure 9.3 Mathematical modelling of RBCs under the increasing shear stress.

9.5 Microfluidic channel designs for aberration-free H^+ iSPIM

In this section, the strategies for microfluidic studies in H^+ iSPIM system are proposed and detailed. The goal is to design suitable microchannels that can be manufactured for use in flow adhesive assays, similar to the RBC and platelet aggregation experiments in chapter 6 and 7. A key requirement of the proposed designs is that the microchannel should introduce negligible or no aberration for both iSPIM and DHM modalities.

From the result of the aberration analysis experiment [Chapter 8.3.3], we concluded that the round tube with a thin wall (0.01mm) introduces negligible aberration for samples within the tube in iSPIM. Furthermore, these capillary tubes are commonly and readily available in the market, increasing ease of adoption. Therefore, the first microfluidic design proposed is a modified round capillary tube. Figure 9.4 demonstrates the three-view drawing and the perspective view of this design. To adapt the round tube to DHM system, a flat

bottom lining is desired to allow samples (cells or platelet aggregations) to stay at the same plane to achieve accurate height map reconstruction. In this case, the normal capillary tube is cut off from one side (the specific proportion of cutting is referred to in figure 9.4) and later is sealed by a thin sheet of PDMS by the plasma treatment method introduced in chapter 4.2.1. The microchannel is then placed on a coverslip with the PDMS side downward and contacted with the coverslip. The PDMS has a good affinity to the glass, which makes the contact seamless and introduces no air between the two surfaces. The background removal process [Figure 5.1.b) in chapter 5] can remove the extra phase delay introduced from the top curved well of microchannels.

Thus, the designed microchannel introduces negligible aberration for both DHM and iSPIM modalities.

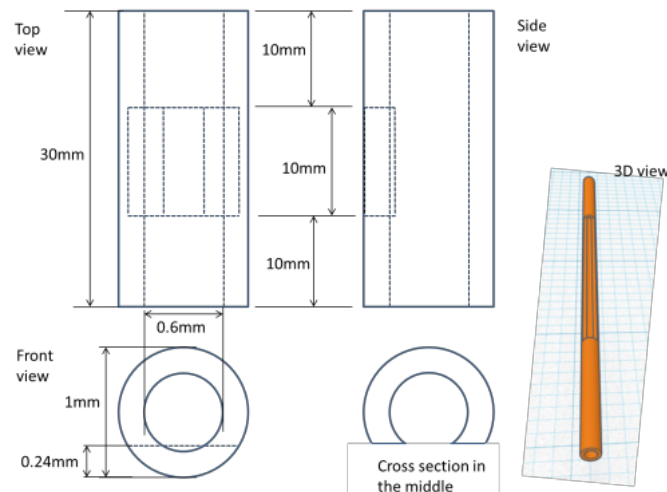


Figure 9.4 Engineering drawing of designed round microchannel from and a 3D view of it.

It should be noted that the aberration analysis of the round microchannel [Chapter 8.3.3] was based on a capillary tube with thin walls of 0.01mm thickness. However, manufacture of such thin tubes brings low yield rate and high cost. Increasing the thickness of the tube wall could increase the reflection of the fluorescent signal which introduces aberrations. Therefore, we designed another polygon tube with 45-degree angle side walls [Figure 9.5]. The flat top and bottom walls provide an aberration-free window for DHM and the two 45-degree angle side walls provide a vertical incident angle for light sheet illumination as well as detection. In figure 9.5, the red dash lines and the red block indicate the

light path of the DHM system. The blue dash lines show the scanning range of the light sheet and detection for the iSPIM system. The green part in the drawing is the FOV that can be achieved by both objectives of the H⁺iSPIM system. Since the illumination and detection in this design are all perpendicular to the tube wall, there is no constraint on the wall thickness as long as the tube wall is transparent. Consequently, the availability of the designed polygonal microchannel can be achieved due to the tolerance of the thick tube wall.

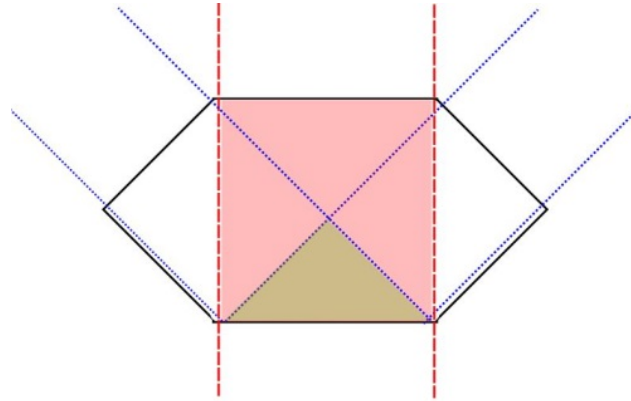


Figure 9.5 Layout of designed polygon microchannel. Red lines represent the laser path of DHM system, while blue lines show the path of laser scanning of iSPIM system.

9.6 Thrombosis study using H⁺iSPIM

The investigation of platelet function with different inhibitors will be the first biological application of H⁺iSPIM, in which the fluorescence images from iSPIM could reveal the actual platelet signalling during aggregations, and the height reconstructions from DHM could provide the morphological and volumetric measurement of the aggregation simultaneously. In this case, the multi-dimensional measurement can be achieved in a single system, which is greatly helpful for exploring the link between biochemical and biophysical aspects of thrombus dynamics. Moreover, Paroxysmal Nocturnal Hemoglobinuria (PNH)²⁹⁻³¹, a rare, life-threatening bone marrow failure disorder, is targeted to be the next subject of H⁺iSPIM study. This is because the high incidence of blood clot formation is the most feared complication in PNH³²⁻³³. While the inhibitor eculizumab is established as the most important treatment for PNH patients³⁴, the mechanism of the pathology is still incompletely understood. H⁺iSPIM, as a multimodality system, could potentially help to provide the comprehensive

quantification of both biochemical and biophysical response of thrombus dynamics with PNH patients.

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Appendix 1

Supplementary for fabrications of microfluidic devices:

Table A1.1 lists out the general procedures of mold photolithography [Chapter 4.2.1] preformed for fabricating the microfluidic devices used in this thesis [Chapter 6, 7].

Step	Procedures
1	Turn on the oven at 120°C.
2	Check all devices (spin coater, hot plate, air gun and UV lamp) and tools (tweezer, 1.5ml syringe, glass dish, petri plate, wast bottle, funnel, pipette, timer)
3	Check all chemicals (photoresist, TMCS, acetone in a bottle, isopropanol, su-8 developer) to the lab with containers
4	Rinse a clean wafer with acetone and dry with pressurized air gun
5	Plasma clean treatment for 15min and 120°C in oven for 15min
6	SU-8photoresist coating for wafer
7	Soft bake with the hot plate
8	UV exposure
9	Post exposure bake with the hot plate
10	Soak the wafer in SU8 developer in a glass dish
11	Rinse with isopropanol and dry with pressurized air gun
12	Silanization (use a few drops of TMCS around wafer inside a petri dish) for 15min and open the dish for 10 min for evaporating
13	Prepare the PDMS and put it in desiccator
14	Turn off all the devices, clean up the fume hood, and dispose general wastes

Table A1.1 General procedure of mold photolithography

From step 6 to step 9, the specific processes are different for fabricating channels with different height. For the microfluidic device used in chapter 6, the height of the microchannel is 25 μm . For the device used in chapter 7, the height of the microchannel is 100 μm . The setting for step 6 to step 9 are listed in table A1.2 respectively.

Step	Procedures	Thickness: 25 μm	Thickness: 100 μm
6	SU-8photoresist coating	3100 RPM for 40s	900 RPM for 40s

7	Soft bake	65 °C for 15 min, then 95 °C for 35 min	65 °C for 15 min, then 95 °C for 2 hours
8	UV exposure	620 mJ/cm ²	1000 mJ/cm ²
9	Post exposure bake	65 °C for 15 min, then 95 °C for 40 min	65 °C for 15 min, then 95 °C for 40 min

Table A1.2 Specific procedures for fabricating microchannel structures with different heights

Appendix 2

Supplementary for Chapter 5

Here we repeated the histogram analysis method in Ref.¹ as shown in figure 5.11 We prepared the sample under a highly diffused medium, a thin film of opaque peptide hydrogel (Fmoc-FRGDF)², which shown in figure A1.1(a). Figure A2.1 (b) shows the detailed breakdown of the histogram selection approaches against the automated region recognition approach as described in figure A2.1 (c). The histogram analysis process requires a Gaussian curve fit followed by two sets of differentiation to retrieve the inflexion point. While it works well in standard samples, it does not operate well on scattering sample as shown in figure A1.1 (b) it shows that the technique cannot accurately retrieve the appropriate phase. For our region recognition, the step increment of the threshold level is set as 1% of GTL and gradually decrease the increment threshold level. During the process, the filtering window progressive enlarges and capture the necessary. When the increment is reduced to 1% of GTL (GTL=0.4627), the reconstruction outcome was shown to display the phase of the microsphere. The caveat of the iterative process is that the first order and zero order must not overlap, which is the limitation of all off-axis DHM systems.

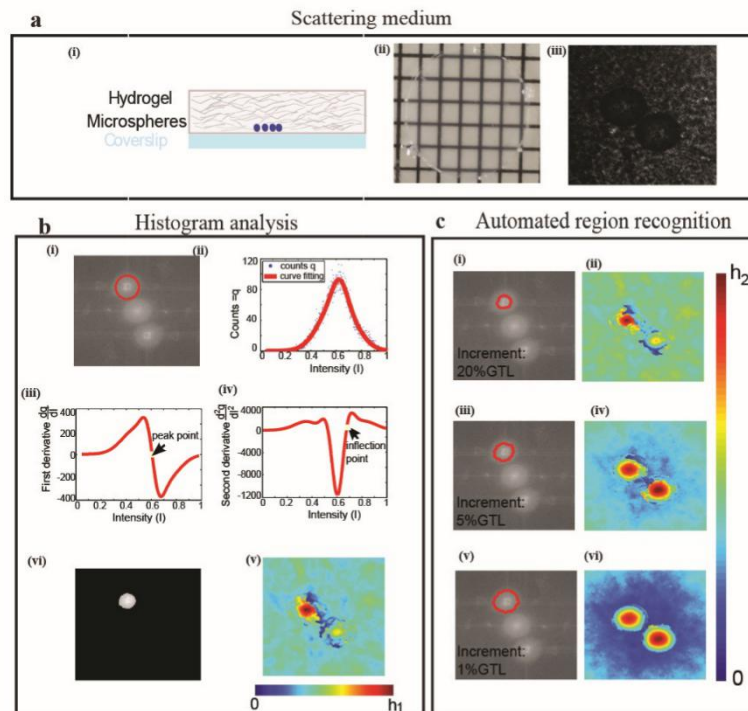


Figure A2.1 Comparison of the phase retrieval process of microsphere embedded in a scattering medium (a) – (i) schematic, (ii) image of scattering medium taken from plan view with grid (iii) acquired hologram. b (i) shows the circle binary filter applied to FFT holograms of the sample. (ii) the histogram of intensity distribution after manually filtering the spatial frequency and Red curve show a Gaussian fit (sum of two Gaussians functions) of the distribution. (iii) and (vi) are 1st and 2nd derivative of the fitting curve in (ii). Inflexion point (intensity =0.67) in iv is used to retrieve the threshold level to obtain the filter in (v) and (vi) and the reconstruction, which insufficiently retrieves the microsphere. (c) increment determination process in automated region recognition method. Figures c(i), (iii), (v) show the different filter windows and (ii), (iv),(vi) are the corresponding reconstructed phase of the microsphere. The threshold windows are created by setting different threshold increment in step (2), Fig.2 (j). Each threshold increments goes from 20% - (i), (ii), 5% - (iii), (iv) and 1% - (v), (vi) of GTL. The results show that 1% of GTL achieves an optimal filter window to retrieve an accurate reconstruction of the microsphere through a diffusive medium. Colour bar $h1 = h2 = 50 \mu\text{m}$.

Here we implemented a second reported histogram techniques³. The crux of the technique is to select a “second” peak in the histogram count. As shown in figure A1.2, their technique operates by applying a negative Laplacian [20] to suppress zero order of spectrum (FFT hologram in our case) and then plotting the histogram of the processed spectrum to locate the “peaks” as shown figure A1.2 (b) and (f). The difference of the intensity of two peaks (intensity of the background and maximum gray-scale point) is then used to allocate the threshold. However, when applying this method to the same samples, there was no sufficient information to clearly locate the second peak (maximum gray-scale point). Figure A1.2 (b) and (f) are the histogram of figure A2.2 (a) and (e). As shown in (b), a second peak in intensity at 0.72 that corresponds to a difference in the threshold to be 0.01 ($0.72-0.61=0.01$). Such low threshold essential covers the entire FFT hologram in figure A1.2 (a) and hence the filter window covers all the spectrum [red rectangle in Figure A1.2 (a)]. This is the same for figure A2.2 (e). Figure A2.2 (c), (d) and (g), (h) are the phase and amplitude reconstructions of two samples under this technique. This method failed to work in locating the right threshold. In such a situation, we needed to manually intervene to insert the threshold values based on intensity of processed spectrums. The proper thresholds become 0.79 and 0.67 as shown in figure A2.2 (i) and figure A1.2 (k) respectively

with desired outcome in figure A1.2 (j) and (i). By relying on the previously reported histogram technique [18], this would mean that the second peak needs to lie at a position larger than the normalized value of 1.

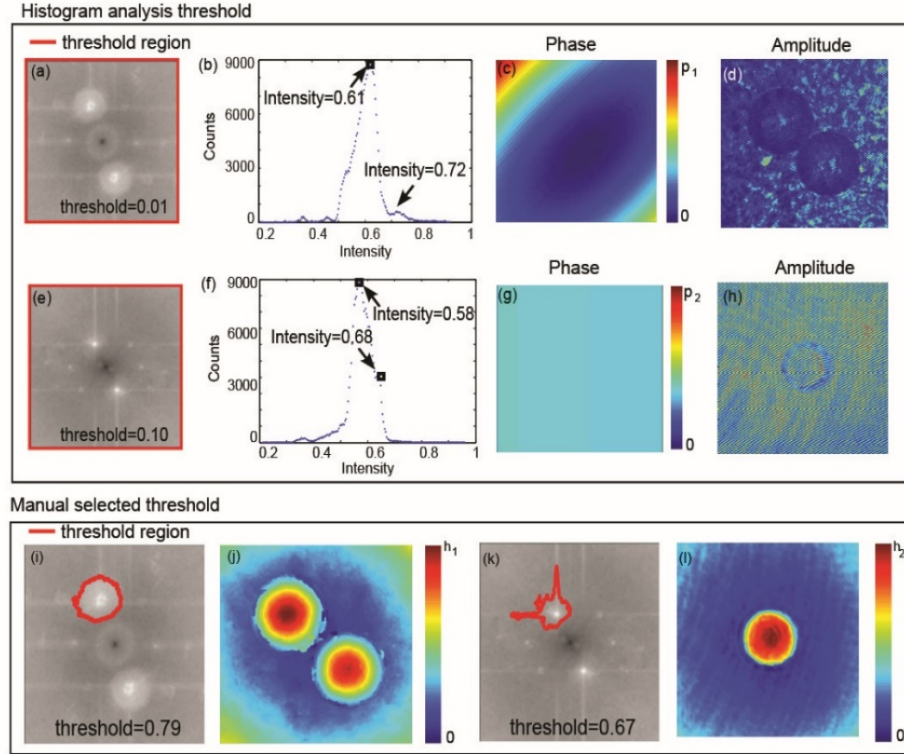


Figure A2.2 shows the detailed comparison of the phase retrieval process of the same sample in Fig 3. The detailed histogram analysis in the previous technique [18] are shown in the top: (a) and (e) are the processed spectrums, in which the red rectangles are the filter windows based on histogram analysis. (b) and (f) are the histograms of (a) and (e) respectively. (c) and (d) are the phase and amplitude reconstructions of (a). (g) and (h) are the phase and amplitude reconstructions of (e). The manual determination of threshold is shown downside: (i) shows the filter window when threshold is 0.79 and (j) is the phase reconstruction of it. (k) shows the filter window when threshold is 0.67 and (l) is the phase reconstruction of it. Colour bar $h_1 = 50 \mu\text{m}$. Colour bar $h_2 = 6.7 \mu\text{m}$. Colour bar $p_1 = 1.0 \times 10^{-4} \text{ rad}$. Colour bar $p_2 = 0.01 \text{ rad}$.

Figure A2.3 gives layout of the double container for biohazard microfluidic experiments [Chapter 6.2.2]. Lines in the figure are used to guide routes for laser cutting using Trotec 360 Flexx Laser Cutter. The material used was clear acrylic (3 mm thickness).

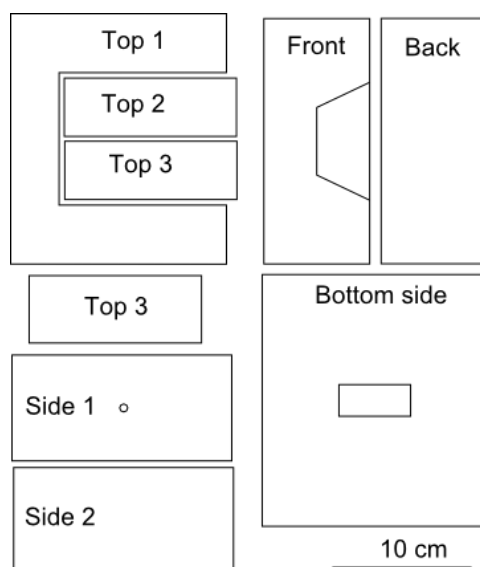


Figure A2.3 Layout of double container design, used for laser cutting.

References:

1. Weng, J.; Li, H.; Zhang, Z.; Zhong, J., Design of adaptive spatial filter at uniform standard for automatic analysis of digital holographic microscopy. *Optik-International Journal for Light and Electron Optics* **2014**, 125 (11), 2633-2637.
2. Li, R.; Horgan, C. C.; Long, B.; Rodriguez, A. L.; Mather, L.; Barrow, C. J.; Nisbet, D. R.; Williams, R. J., Tuning the mechanical and morphological properties of self-assembled peptide hydrogels via control over the gelation mechanism through regulation of ionic strength and the rate of pH change. *RSC Advances* **2015**, 5 (1), 301-307.
3. Li, J.; Wang, Z.; Gao, J.; Liu, Y.; Huang, J., Adaptive spatial filtering based on region growing for automatic analysis in digital holographic microscopy. *Optical Engineering* **2015**, 54 (3), 031103-031103.

Appendix 3

Supplementary Figures for Chapter 7

Figure A3.1 – distribution of thrombus peak height.

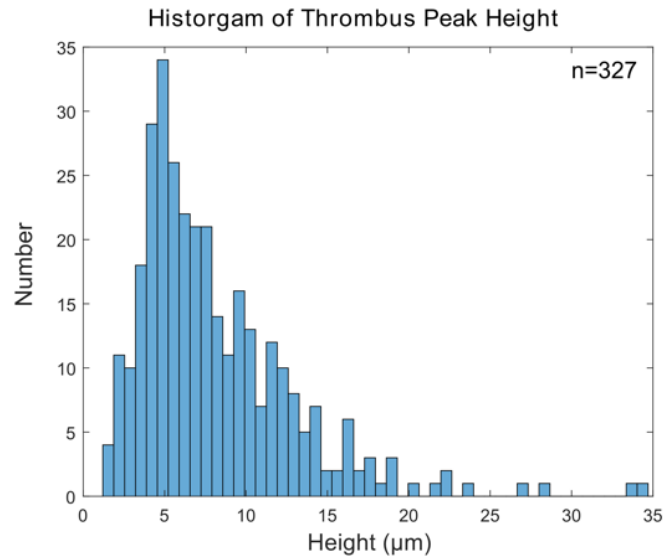


Figure A3.1 Histogram of thrombus peak height. Thrombus (n=327) were formed by the PRP from all donors (D1, D2, D3, D4 for citrated PRP and d1, d2, d3, d4 for heparin PRP) under all three experimental shear rates.

Figure A3.2 – Volume profiles for thrombus formation using platelet rich plasma (PRP)

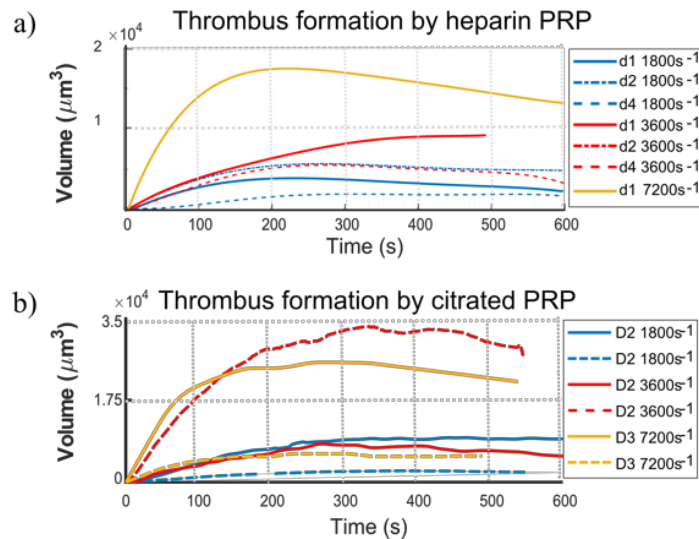


Figure A3.2 a) Volume growth of thrombus formed by heparin PRP under three different shear rates. b) Volume growth of thrombus formed by citrated PRP under three different shear rates. Each line stands for each thrombus. The colour of the lines indicates the shear rates of flowing PRP, blue for 1800 s⁻¹,

red for 3600 s⁻¹ and yellow for 7200 s⁻¹. The type of lines distinguishes the thrombus from different donors.

Figure A3.3 – Stability test of thrombus formed by citrated PRP

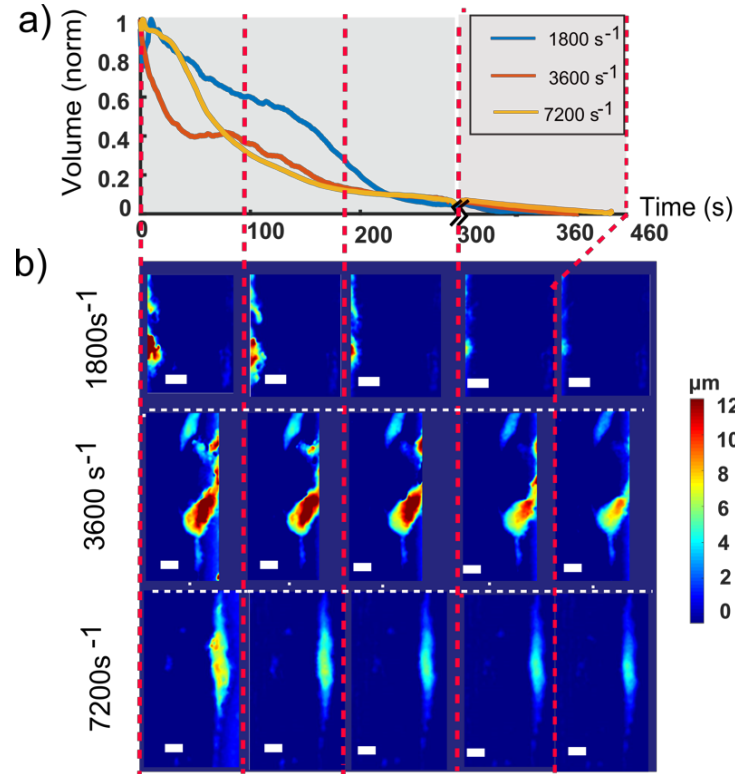


Figure A3.3 a) Volume reduction of thrombus formed by citrated PRP during stability test (buffer flowing at 7500 s⁻¹ for 5 min and then 12500 s⁻¹ for 2 min). Each line profile traces the thrombus volume that was previously formed under shear rate of 1800 s⁻¹, 3600 s⁻¹ and 7200 s⁻¹ from donor D2 respectively. b) The height map images below the line plots are the corresponding thrombus at different time points. The scale bar is 30 μm and colour bar is in micron.

Appendix 4

Supplementary for Chapter 8

This section shows the characteristic of holograms from H⁺iSPIM in chapter 8. The height of the fibre tip [Chapter 8, Figure 8.2] can be controlled by a post clamp employed in H⁺iSPIM system. As the two objectives for iSPIM require water immersion, the end of the bare fibre can be either immersed in the water or upon the water surface. We evaluated holograms taken from both situations and the results showed both situations provided the high quality of interference patterns (fringes). Weber Contrast was used to indicate the quality of the fringes. The equation of the contrast calculation is according to $C = (I_{\max} - I_{\min}) / I_{\min}$, where I represents the intensity of the pixels in holograms. When the end of the fibre is outside the water [Figure A4.1 (a)], obtained hologram possesses fringe of around 0.6 contrast. While the fibre is immersed in water, the contrast of the fringes is around 0.7 [Figure A4.1 (b)]. Both situations can generate holograms with clear interface fridges.

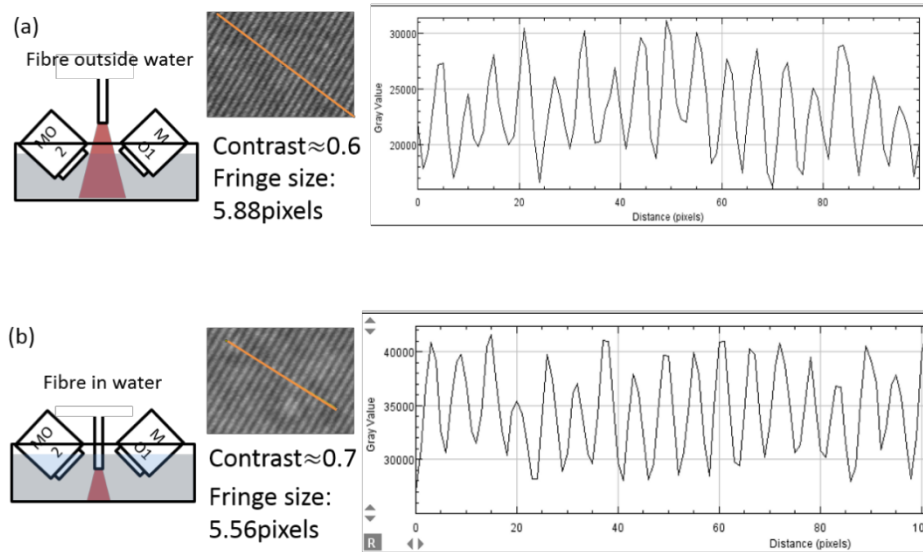


Figure A4.1 Evaluation of image contrast of interference patterns under two situations. (a) indicates the situation that the fibre is placed upon the water surface. The small region of hologram taken in this situation and the intensity profile of the fridges in hologram is shown beside. (b) indicates the situation that the fibre is immersed in water. The small region of hologram taken in this situation and the intensity profile of the fridges in hologram is shown beside, referred to the yellow line over the hologram.

