Chapter 1

Introduction

Over the last several decades, biomedical imaging has undergone a significant and a rapid technological advancement and has witnessed development in many new and major applications [1,2]. Various techniques namely functional imaging [3,4], spectroscopic imaging [5], optical imaging [6,7] etc are being embraced and incorporated in various spheres that range from fundamental research to clinical applications, as well as from cellular level to the whole-organ level. Biomedical imaging has changed the face of healthcare industry by allowing medical practitioners to learn in depth about the human anatomy and various diseases. Moreover, biomedical imaging being an interdisciplinary field, has proven to be beneficial as an approach to obtain and interpret crucial details from an extensive database in various fields. This information has played a very critical role in clinical investigation by assisting with the quick diagnostics thereby monitoring, preventing and controlling various aspects associated with health conditions [1,8,9]. It is expected that the application of biomedical imaging along with artificial intelligence will help in the advancement of image interpretation which will eventually assist in better disease prediction and thereby therapy planning [10].

Although the field of biomedical imaging has evolved in leaps and bounds and has become a pivotal in the care pathway of various ailments, there is a major shortage of imaging equipment and workforce in low-and-middle income countries which can be considered as an obstacle for the quick and affordable diagnosis and treatment of several diseases such as malaria, tuberculosis, sickle cell anaemia, etc. Millions of deaths occur mainly due to the lack of diagnostic facilities, out of which many of the fatalities are of children under the age of five [11]. As per the report published by the World Health Organization (WHO) in the year of 2017, 216 million people suffered from Malaria out of which 0.44 million cases were proven to be fatal [11–13]. Yet another report from WHO published in the year 2020 suggests that 0.43 million people succumbed to Malaria alone in the year 2015 [14]. Anaemia, a disease which is caused due to an imbalance in the iron content in the body, afflicts the human health significantly, causing death in rare cases. Major portion of the population in the under developed countries are affected by it [15]. Around 1.6 billion people are anaemic worldwide [16,17]. Similarly, there are several potentially fatal diseases which are prevalent in developed as well as under-developed countries [18]. Proper treatment of such diseases in the under developed and developing countries, requires quick and affordable diagnostic tools. Moreover, these diagnostic tools are required to be operated under harsh/stringent conditions with minimum human intervention, therefore the tools need to be rugged, stand-alone, compact as well as inexpensive.

Human erythrocytes also known as Red Blood cells (RBCs) provide the information about the state of health of the human body. Diseases such as sickle cell anemia, malaria etc. leave their trace on the cell morphology of RBCs [19–23]. Quantifying the mechanics of the RBCs provides more sensitive information of their structure and might suggest new insights into the etiology of several diseases. Therefore, understanding the changes in the rheology of the blood as a result of modulation of the RBC's morphology and mechanics may contribute to the understanding of these diseases. [24–35].

One of objectives of the work presented in the thesis is imaging and optical characterization of cells which might be useful for disease diagnosis. As discussed above, it is already been reported that the RBCs carry the imprints of some of these diseases [19–22]. Moreover, it has been observed that RBCs exhibit spontaneous membrane fluctuations or "flickering" [2,36] which were first observed a century ago. These thermally induced submicron motions are identified by membrane displacement or membrane motions. These submicron motions take place in the millisecond scale or less (with 100nm scale amplitudes at frequencies of tens of Hz). Investigating these nano-meter scale cellular dynamics prove to be highly relevant in furnishing crucial information about the mechanical properties of the membrane-cytoskeleton ensemble [27,35,37–39].

There are several objects, including most of the living cells, that do not attenuate the intensity of the electromagnetic radiation passing through them significantly thereby giving rise to low contrast two-dimensional image in a conventional bright field imaging system [40]. Such objects are called phase objects. RBCs do share these characteristics of the phase objects and they provide low contrast two dimensional images under a bright field microscope [41–44]. The contrast can be improved by staining the sample, which involves treating the sample chemically. However, staining the sample might lead to undesirable alteration in the structure of the cells or its constituents [45].

Imaging modalities such as confocal and fluorescence microscopy [46–49] can also be brought into practice, to improve contrast, quantification of cell morphology and localization of the cell structures. However, these techniques require chemical treatment and scanning of the sample which makes them cumbersome forbidding real time inspection and study of certain dynamic events. Besides, it also requires a trained technician for staining the sample, data acquisition, and data analysis.

Contrast can also be improved by translating the phase change occurring in the probe beam traversing through the sample, into a change of amplitude, leading to phase contrast imaging. The recorded phase may also be utilized to determine the thickness profile of the specimen which provides valuable information about the sample [50–63].

1.1 Methods to perform phase contrast imaging

Zernike's and Nomarski's methods are the two most widely used techniques[40,50] for performing quantitative phase contrast imaging (QPI). But, due to shading-off effect present in the Zernike phase contrast microscopy, quantification of the phase contrast cannot be obtained. Another technique that can be used to perform QPI is the Differential interference contrast (DIC) method also known as Nomarski technique. However, some sophisticated methods for signal acquisition and processing are required for converting the intensity images into quantitative phase distribution. Interferometric Microscopy has emerged as a powerful mechanism for quantitative phase imaging of biological samples [64,65]. Amongst various interferometric methods, off axis Digital Holographic microscopy [66–69]; a state-of-the art technique, has gathered immense attention since it is a single shot imaging technique which puts forth an excellent approach for performing quantitative phase imaging and offers a number of significant advantages such as direct access to the phase of the sample wavefront from which the thickness information can be computed and numerical focusing allowing information to be retrieved from various axial planes. This label free quantitative phase imaging technique has the ability to acquire holograms videos, permitting the study of dynamic events leading to extraction of bio-mechanical properties along with the bio-physical properties of biological samples [22,52,54,56,57,60,64,65,68–84].

Digital Holographic Microscopy can be performed using various geometries depending on the application, stability, compactness, cost effectiveness, and field portability of the setup.

1.2 Gabor Digital holographic Microscopy

Dennis Gabor in 1948 invented "a new microscopic principle" which he termed "holography" as a "method for recording and reconstructing the amplitude and phase of wavefields" [66,85–89]. Dennis Gabor was awarded with the Nobel Prize in Physics in the year 1971 "for the invention and development of the holographic method”. In the original setup, the reference wave and the object wave were located along normal axis with respect to the photographic plate, which was used as the hologram recording medium. The source beam (spherical and diverging) illuminated the sparsely distributed semi-transparent object. As shown in Fig 1.1, a single illuminating beam was used. The illuminating wave gets partly scattered due to the sparsely distributed object which acts as the object beam. The un-scattered beam, which is devoid of object information acted as the reference acts as a coherent background (reference). The partially scattered beam that carries the object information and the reference beam interfere at the hologram recording medium (digital array in the case of digital holography). Since both reference and object beam proceed along the same centre line (no angle between them), Gabor geometry is also called in-line geometry. In digital holographic microscopy, the holograms are recorded on digital arrays like CCD or CMOS array. They are reconstructed by carrying out the simulations of the diffraction of the digitally inputted reference wave from the hologram structures by numerically implementing the diffraction integrals [67].

Gabor digital holographic microscopes are easy to implement due to its simple structure and a compact as well as cost effective microscope can be developed. However, this geometry is more suitable for those objects which are sparsely distributed as most of the beam reaching the detector plane should remain unperturbed. Moreover, this method suffers from “twin image” problem which is the superposition of a focused real image of the object, a de-focussed virtual image and an un-diffracted DC term [66]. These diffracted beams are inseparable using conventional in-line techniques. As a result of the “twin image” the object cannot be reconstructed unambiguously because of the unwanted artifact term which could potentially lead to a greater error in locating objects along the direction of the optical axis as well as in correct extraction of object phase information. Efforts have been made in the direction of dealing with the twin image problem by applying numerical methods which at times can be computationally exhaustive [90]. Such technique may also require recording of multiple in-holograms and the numerical reconstructions of the object wavefront are based on iterative algorithms, requiring priori knowledge about the object [90].

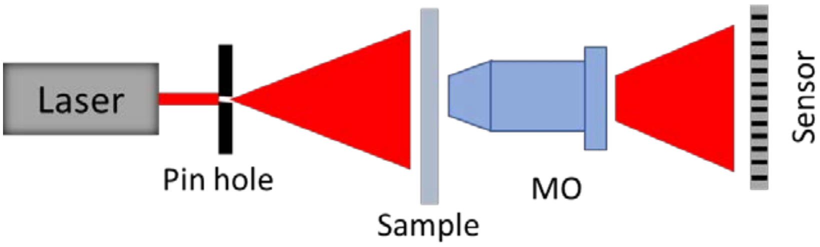

Fig 1. 1 Schematic of in-line digital holography configuration

1.3 Off-axis Digital Holographic Microscopy

To overcome these drawbacks (sparse object distribution and twin image problems), Leith and Upatnieks inserted an off-axis reference wave in such a way as to make the reference and the object beam interfere at an angle at the detector plane [66,91,92]. Such a configuration eliminates the “twin image” problem as it separates out the real, the virtual and the un-diffracted beam in both spatial as well as frequency domain. The offset angle between both the beams adds a carrier frequency to the signal which makes the unambiguous numerical reconstruction of the object beam possible. The off-axis geometry eliminates the twin image issue as Fourier Transform of digitally recorded holograms can be taken numerically and desired part of it can be filtered to extract only the object information [66]. As shown in Fig 1.2, an off-axis configuration (Mach Zehnder interferometer), the incoming beam is divided into two identical beams. Out of the two split beams, one of them trans-illuminates the object under examination called as an object beam, while the other beam remains unperturbed known as the reference beam. Both the beams interfere on the digital array sensor which is nothing but the image plane of the lens used to magnify the object. The optical path travelled by both the beams must be almost equal to generate the interference fringes or the hologram.

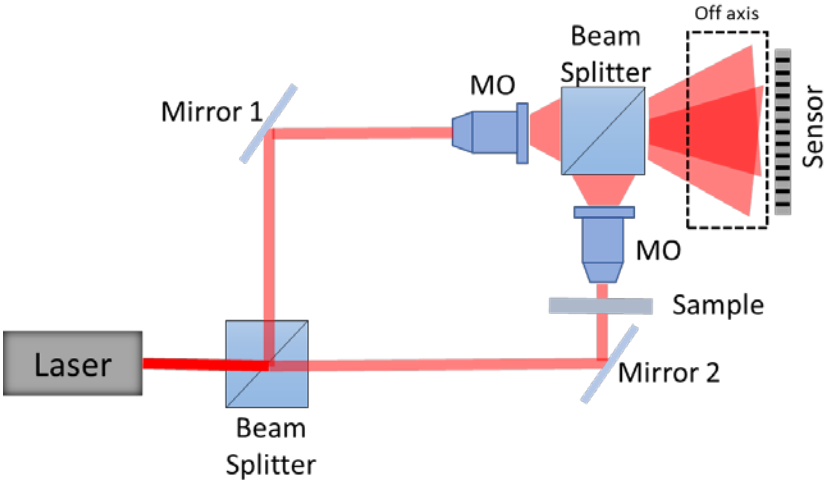

Fig 1. 2 schematic of Mach-Zehnder interferometer configuration for digital holographic microscopy

1.4 Two beam off axis and common path geometries

Off axis configuration has two types:

(1) Two beam geometry (For example: Mach Zehnder)

(2) Common path self-referencing geometry (For example: Lateral shearing)

The Mach-Zehnder configuration (Fig. 1.2) employs a beam splitter to split the source beam into two beams called as object and reference beam. Both the beams are reflected with the help of mirrors and a second beam splitter helps them superimpose at the sensor plane. The sample arm contains the sample and a Microscope Objective (MO) which is a finite conjugate objective is placed to magnify the object. This geometry is also referred to as Linnik interferometry. While the reference arm which is devoid of any object information passes through a MO of similar configuration as that of the object beam. This MO is placed in the reference arm to match the wavefront curvature produced in the object arm to generate linear fringes at the hologram plane. The holograms are recorded by the digital sensor and the object information is numerically reconstructed [58,66,69].

In the Mach Zehnder configuration, both reference and object beams travel along different paths. This might lead to the reduction in the temporal phase stability of the microscope, which in turn may affect its use in the measurement of cell thickness fluctuations of the order of few tens of nanometer, for example, in the case of human erythrocytes. The uncorrelated path length variations make the system susceptible to acoustic noise and air turbulence which can also lead to difficulties in real-time phase measurement. Hence, it becomes difficult to undertake study of dynamic events by employing these geometries [64,65,80]. A vibration isolation mechanism can be integrated in the system to improve its temporal stability but it will increase the cost and bulkiness of the system. Moreover, this geometry is not the most favourable when the portability of the system and ease of manufacturing a working model is concerned, as it employs multiple optical elements for beam steering, beam splitting and beam combining which significantly affects its cost and size. Furthermore, the complexity of this configuration prevents the use of low coherent sources as it requires accurate adjustment of path lengths.

Both the in-line and off-axis geometries usually employ a laser source for the ease of holographic fringe generation. However, laser being a coherent source, induces various unwanted effects that ultimately degrade the image quality. These effects include formation of parasitic interference fringes that originates due to multiple reflections/transmission from different location of the system and generation of speckle noise due to the scattering [88,93–101]. To reduce these noises, quasi monochromatic sources such as LEDs can be incorporated in these geometries. Moreover, in comparison to Laser diodes, LEDs can generally be cost effective, have larger emitting regions and have a longer lifetime [102].

Investigations have been carried out for reducing the spatial coherence of the laser by using a moving diffuser [93,103–112]. A study has also been reported on the influence of partial temporal-coherence on holography [93,99,100,113–120]. The concept of using a rotating ground glass is applicable to tailor the spatial coherence according to the requirement, but it becomes impractical to have a miniaturized and portable setup with high temporal stability [93]. The partially coherent source may solve the problem of coherent artefact noise in the two beam off-axis geometries but it becomes extremely difficult to adjust the path length difference to produce high contrast interference fringes over a large field of view (FOV). Moreover, partially coherent source cannot help in reducing the number of components and the cost of manufacturing of the two beam off-axis geometries.

To reduce these noises, quasi monochromatic sources can be incorporated in these geometries. Modern narrow band LEDs are quasi monochromatic sources, which can also be considered as a source having low temporal coherence. Temporal coherence of any source is associated with the intrinsic bandwidth of the source, whereas the spatial coherence is related with the size of the source and the distance it travels [98]. Spatial coherence can be engineered to enhance the interference effects, making LEDs instrumental to manufacturing of compact, robust and inexpensive off axis digital holographic microscopes[114,117,121,122]. However, construction of off-axis digital holographic microscopes using two beam geometry (Fig. 1.2) employing LEDs is extremely challenging as it requires micrometer level adjustment of pathlength difference between object and reference beams.

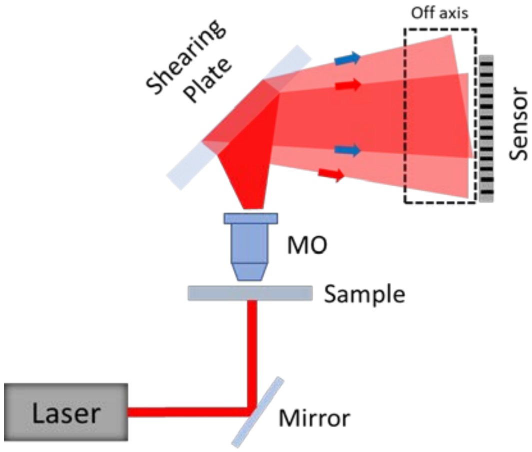

Fig 1. 3 Schematic of Lateral shearing geometry

1.5 Common Path Self-Refencing Digital Holographic Microscopy

Off-axis digital holographic microscopy can also be achieved by employing self-referencing geometry. In such a microscope a single beam illuminates the object. The portion of the object wavefront (devoid of object information) interferes with the portion of the same wavefront containing object information by employing amplitude division or wavefront division interferometer modules [52,58,64,65,80,82,84,123–126]. There are various common path self-referencing geometries for performing off-axis digital holographic microscopy which allows the microscope to achieve high temporal stability owing to their simple and compact arrangement [52,58,64,65,80,82,84,123–126]. Self-referencing interference fringes can be obtained by using lateral shearing geometry. The lateral shearing interferometer is a common-path self-referencing geometry that uses a shearing plate to split the incoming beam into two beams. Although both the beams contain the object information, the unmodulated portion of one beam out of the two is used as a reference for the object information present in the other beam [58,65,84]. Fig 1.3 represents a schematic of the digital holographic microscope based on the lateral shearing interferometer where a thick glass plate (shearing plate) reflects the beam from both its front and back surfaces to generate two laterally sheared beams which are made to interfere at the sensor plane. Other than lateral shearing, there is radial shearing and rotational shearing as well [127,128].

Although high stability is achieved, integrating LED in lateral shearing configuration, which is a common path self-referencing geometry, is difficult, because the path length difference (generated by thickness of the plate used is of the order of few millimetres) between both beams may not fall within the coherence length of the LED (order of few micrometre) to achieve required interference pattern over large FOV. Even if stable interference pattern is achieved, the coherence is not enough to provide appreciable visibility of fringes over a larger area restricting FOV only to a very small area. Thus, an off-axis digital holographic microscope which is temporally stable, and employing an LED source and providing a larger FOV by tailoring the coherence properties of the source will be highly appreciated [129,130].

To develop a cost effective and a compact digital holographic microscope, the configuration implemented should be able to use temporally low coherent sources by utilizing a special common path self-referencing geometry, providing a large FOV.

Work presented in this thesis, explored various common path self-referencing digital holographic geometries such as Sagnac, Lloyd's mirror and Fresnel Biprism employing LED for examining bio-physical and bio-mechanical parameters of biological specimen mainly human RBCs.

1.6 Fringe Projection Technique

Quantitative phase imaging of phase objects could also be achieved without interferometric methods. One of these methods uses projecting a structured pattern (fringe pattern) through the sample and measuring the change of the pattern from the expected one [131–134]. Fringe projection technique is considered non-interferometric and non-invasive in nature which is used in optical metrology. It can be broadly divided into three sections: (1) Projection unit (2) imaging unit and (3) processing unit. The projection unit projects a known fringe pattern onto the object under investigation while the imaging unit captures the fringe pattern modulated by the object information. The processing unit analyses the recorded pattern and computes the change in the fringe pattern upon inter?action with the object and this change in the fringe pattern is converted into phase information (gradient phase) using fringe analysis technique. This technique can be used for studying the three- dimensional shape of objects [131]. Fig 1.4 shows the block diagram of the Fringe projection technique.

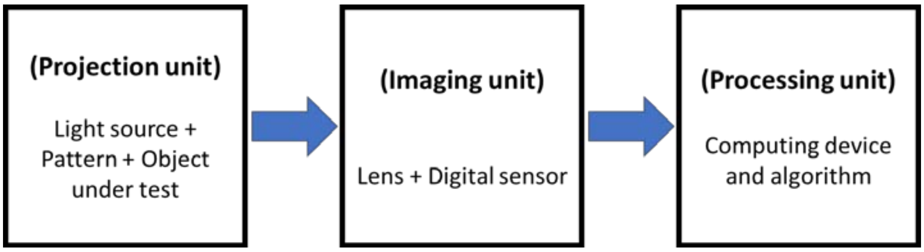

Fig 1. 4 Block diagram of Fringe projection technique

This method offers the following features: (1) easy implementation; (2) phase shifting, fringe density and fringe orientation change can be carried out with no moving parts; and (3) fast full field measurement (4) privilege to use any light source. Owing to these merits, the fringe projection method has been commercialized in the coordinate measurement and machine vision industries as many promising applications can be found [135,136].

The work presented in this thesis aims at developing a simple and compact arrangement based on fringe projection technique for the shape measurement of transparent optical components such as wedges, lenses and cylindrical rods and its application towards investigation of micro-objects including human red blood cells using a linear fringe pattern printed on a paper. The printed pattern is projected through the object under investigation using an LED source and a projection unit. The pattern modulated by the object is analysed to quantify the sample under test. A huge disadvantage of the Fringe projection method is that it is based on triangulation which requires a bulky setup. Moreover, compared to interferometry the fringes aren't actually located on the object surface, whereas speckles in holography or speckle interferometry are, so it is much easier to correct for any displacement that may occur between two temporally consecutive recording.

1.7 Large field of view self-referencing lens-less Holographic Microscopy

To attain high-resolution imaging lenses are necessary, nevertheless lens-less [137–141] imaging is serviceable where high resolution is not a major requirement. The lens-less setups have already been used for different imaging applications for bio-pharmaceutical applications, in air quality monitoring [139]. Chromatic and wave aberrations due to lenses and other optical components distort the reconstructed image that is a tedious task to remove it computationally [142]. Also, a small FOV is obtained using the systems equipped with magnifying lenses (due to optical magnification). To remove all the above discrepancies an off-axis lens-less approach for performing microscopy would be suitable to obtain large FOV, especially where the requirement of high resolution can be compromised. Fig 1.5 shows the block diagram for the lens-less digital holographic technique.

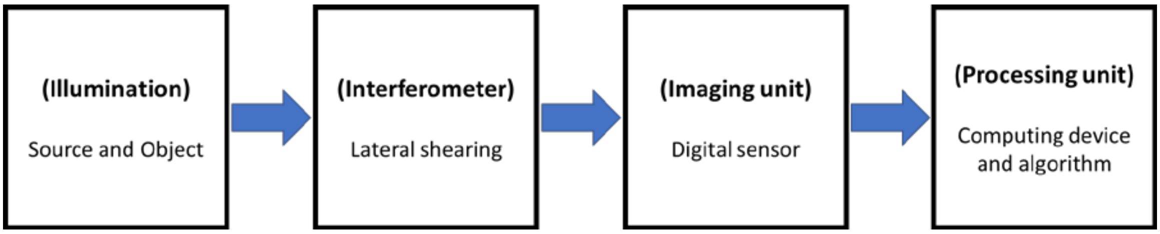

Fig 1. 5 Block diagram of Lens-less digital holographic technique

An off-axis, lens-less, self-referencing microscope that can image biological samples, which is easy to operate and deployable in remote locations is developed. In the developed microscope Laser diodes are used as illumination sources, which works on the principle of self-referencing digital holography. LEDs can also be used as a source of illumination but only using specific geometries owing to its low temporal coherence. High-resolution target such as USAF and amplitude objects (ink dots made on microscope slide) have been imaged using the microscope.

1.8 Low-cost Optical Coherence Tomographic System using LEDs

In the last decade, methodology for imaging human tissue has seen substantial progress ranging from basic histopathological analysis under a conventional microscope to recent machine learning assisted image processing and analysis based quantitative approach [143]. Medicine and healthcare in general are experiencing a paradigm shift, moving from reactive to proactive approaches [144]. Innovative imaging technologies are providing researchers with the power to visualize, characterize and quantify biological and molecular phenomenon with a hitherto unattainable precision [145,146]. It is crucial to examine and understand the structural and functional aspect of tissues which helps in characterizing the condition of the tissue and thereby detecting and quantifying abnormality present. Understanding the underlying cause of the abnormality can provide a better insight of several diseases associated with tissues. It also helps in designing the efficient plan of action for its treatment [147–152].

One of the limitations of interferometric technique in the transmission mode is that it cannot be employed in imaging thick samples or biological tissues as optical radiation passing through the tissue gets significantly scattered, which leads to image degradation. This restricts the use of this technique only in imaging thin biological samples where the thickness is lesser than the mean free path for scattering [153,154]. To tackle this problem Optical Coherence Tomography (OCT) is employed for performing the three-dimensional sub-surface, non-invasive imaging of biological tissues which makes use of a broadband source [149,155–157]. While examining inhomogeneous samples (example: biological tissues), OCT proves to be useful since it produces high-resolution cross-sectional images of inhomogeneous samples. It also finds variety of applications in the fields like ophthalmology, gastroenterology, dermatology, and cardiovascular imaging, etc.[153,154,158–161]. Optical Coherence Tomography has been materialized as a rapid, non-invasive and non-contact optical imaging technique which offers cross sectional images of biological tissues and it makes use of low coherence interferometry[161,162].

A low-cost Fourier Domain Optical Coherence Tomography (FD-OCT) system which employs a Light Emitting Diode (LED) owing to its relative simplicity, compactness, robustness, and low cost, instead of Super luminescent Diode (SLD) that is the standard light source used in commercially available OCT devices is designed and developed. The device also used low-cost imaging sensors like Webcam instead of a high-end detector, reducing the cost further.

1.9 Summary of the Thesis

The work described in the thesis focuses on incorporating LED for performing quantitative phase contrast microscopy using both interferometric and non-interferometric techniques for examining human erythrocytes also known as Red blood cells (RBCs) as well as employing LED in optical coherence tomography for obtain sectional images and making the system overall cost effective.

1.9.1 Chapter 1: Introduction

Chapter 1 puts forward an introduction and overview to the problem that is addressed in this thesis. It provides a brief of the techniques involved in the work and a short discussion and introduction to each technique including their pros and cons. Non interferometric and interferometric techniques have been discussed which have been implemented to perform quantitative phase contrast imaging of RBCs. A lens-less technique has also been discussed which has been employed to reduce the aberration that arise due to lens and other optical components. FDOCT has also been performed utilizing LED instead of the conventional light source used which is the superluminescent diode in order to make the system cost effective.

1.9.2 Chapter 2: Coherence

Chapter 2 discusses about the fundamental and inherent property of the light source; coherence, which has a particularly important implication in imaging systems. Broadly speaking, coherence has two aspects associated to it, one that focuses on the correlation of a wave with itself at different time points is termed as temporal coherence whereas the one that represents the mutual coherence of different parts of the same wavefront termed as spatial coherence The chapter describes both temporal and spatial coherence and how the use of laser (high coherent source) poses some limitations in the imaging system by degrading the image quality (due to speckle noise). It also discusses several advantages of utilizing LED (low coherent) as an alternative to laser in interferometric as well as non-interferometric techniques which are digital holographic microscopy and fringe projection technique respectively.

1.9.3 Chapter 3: Fringe Projection Technique

Chapter 3 focuses on the implementation of a non-interferometric, non-invasive technique for generating three-dimensional surface information namely Fringe projection technique which is made up of a projection unit, image acquisition unit and a processing unit. In this technique, a structured pattern (sinusoidal or grid pattern) is projected on the object under investigation. Depth information is encoded in the deformed pattern which is imaged by the digital sensor array. This technique can be used in two modes: reflection and transmission. The study focuses on shape measurement of optical components such as wedges, cylindrical rods and cylindrical lens etc. Further the technique is also implemented to retrieve three-dimensional information of human RBCs.

1.9.4 Chapter 4: Theory of holography

Chapter 4 explains the theory behind the recording and reconstruction of holograms. It also describes the mathematical formulation related to the angular spectrum propagation approach of the scalar diffraction theory and Fourier fringe analysis that is used to analyse the recorded data.

1.9.5 Chapter 5: Wide field of view common path self-referencing digital holographic microscopy employing LED

Chapter 5 describes how low coherent source such as LED is integrated as a light source in various common path self-referencing geometries to perform quantitative phase contrast imaging of human RBCs. The coherence properties of LED are exploited in order to generate high contrast interference fringes over a large FOV. Common path self-referencing configuration such as Sagnac, Lloyd’s mirror and Fresnel biprism have been explored to harness the coherence of LED employing it for performing digital holography microscopy. The work also includes increasing the effective FOV by hologram multiplexing. The use of exotic wavelength such as UV LED has also been explored for performing the experiment in so as to enhance the resolution of the system. The chapter also includes designing and development of 3D printed, stand alone, portable and cost-effective device based on Lloyd's mirror interferometer and Fresnel Biprism interferometer. A comparative study has also been undertaken using the above-mentioned geometries and with a combination of different sources and sensors.

1.9.6 Chapter 6: Large field of view self-referencing lens-less Holographic Microscopy

Chapter 6 describes the application of lens-less imaging techniques. To attain high-resolution imaging, lenses are necessary nevertheless lens-less imaging is serviceable where high resolution is not a major requirement. A lens-less digital holographic microscope is developed to examine micro- objects by employing lateral shearing interferometer. Furthermore, the lens-less system would make the microscope compact, easy to implement, portable, robust, and also eliminate the aberration introduces due to a lens.

1.9.7 Chapter 7: Low-cost Optical Coherence Tomographic System using LEDs

Chapter 7 introduces the theory of Optical coherence tomography (OCT) which is a rapidly emerging, robust, non-invasive, three-dimensional sub surface tissue imaging technique. In this chapter design of a cost-effective Fourier Domain Optical Coherence Tomography (FD-OCT) system which employs an LED source is describes. The LED source offers features such as relative simplicity, compactness, robustness. Moreover, LEDs are low cost, unlike Super luminescent Diode (SLD) which is the standard light source used in commercially available OCT devices. The developed design uses a Webcam instead of a high-end detector to record the data. Theoretical formulation of Time domain OCT and Fourier Domain OCT has also been discussed in this chapter.

1.9.8 Chapter 8: Conclusion and future scope

Chapter 8 concludes the outcomes of the thesis and discusses the future prospects of utilizing exotic wavelengths in performing digital holographic microscopy.