Chapter 1

Introduction

Millions of deaths occur in developed, developing and under developed countries due to lack of diagnostic facilities, especially remote diagnosis devices. In 2016 alone, 216 million people suffered from malaria out of which 445000 cases were fatal [1]. Most of these cases occurred in the under developed nations of Africa. Many of the fatalities were children under the age of five [1]. More than one-third of the world population is infected with tuberculosis and in 2016 [2]. It causes ill-health for more than 10 million people every year and is one of the top causes of death worldwide [2]. Similarly, there are several potentially fatal diseases which are widespread especially in developing and under-developed nations. Proper treatment of these diseases first requires their correct diagnosis. To achieve this, in developing and under-developed nations with vast population, one requires diagnostic tools that could be operated by untrained, non-technical personnel, requiring minimum human intervention. Large population also necessitates deployment of large number of diagnosis devices. So, the diagnosis devices should be lower in cost and rugged. Also, for on-field applications, one requires stand-alone devices which are smaller in size so that they can be transported easily. The main focus of the work presented in this thesis was to design, develop and fabricate low-cost, compact devices, based on the principle of interaction of electromagnetic radiation with the sample under investigation.

1.1 Microscopy

Most of the cells, which provides information about state of health of an individual cannot be seen with naked eye. It requires the use of a lens to magnify the sample (cells in our case) so that it become visible. This leads to the field of microscopy [2-8]. Since its invention, microscopy proved to be the most vital technique in the monitoring, characterization and identification of cells and other micro-objects. Microscope is one of the most influential analytical tool in a vast range of scientific disciplines including medical sciences. Microscopes allow scientists and researchers to study microscopic objects around them which are invisible to the naked eye and it led to encounter existence of the microorganisms, bacteria, fungi, learning the structure of plant or animal cells and identification of diseases.

Based on contrast enhancing principle and method of operation, many microscopy techniques (different modalities) are available to increase the specimen contrast and information throughput. These include, bright field microscopy, fluorescence microscopy, confocal microscopy, phase contrast microscopy, super resolution techniques, iterative phase retrieval techniques etc [2-8]. All these techniques have their own advantages and disadvantages.

1.2 Bright field microscopes

Most widely used technique involves illuminating the sample with a broadband light source. This leads to bright field microscopy. This technique still stands as a gold standard technique for diagnosis of many diseases and illness in hospitals and clinics as well as primary examination method for microscopic samples. Bright-field microscopy is the simplest and easiest technique which can be achieved by single lens and a screen (or imaging sensor) for imaging of microscopic objects. The main function of microscope is to create magnification with proper resolution and contrast, so that it is possible to differentiate between small micro structures based on its absorption profile [3-4]. Fig. 1.1 shows the intensity images (bright field images) of red blood cells illuminated by a light source working at 635nm.

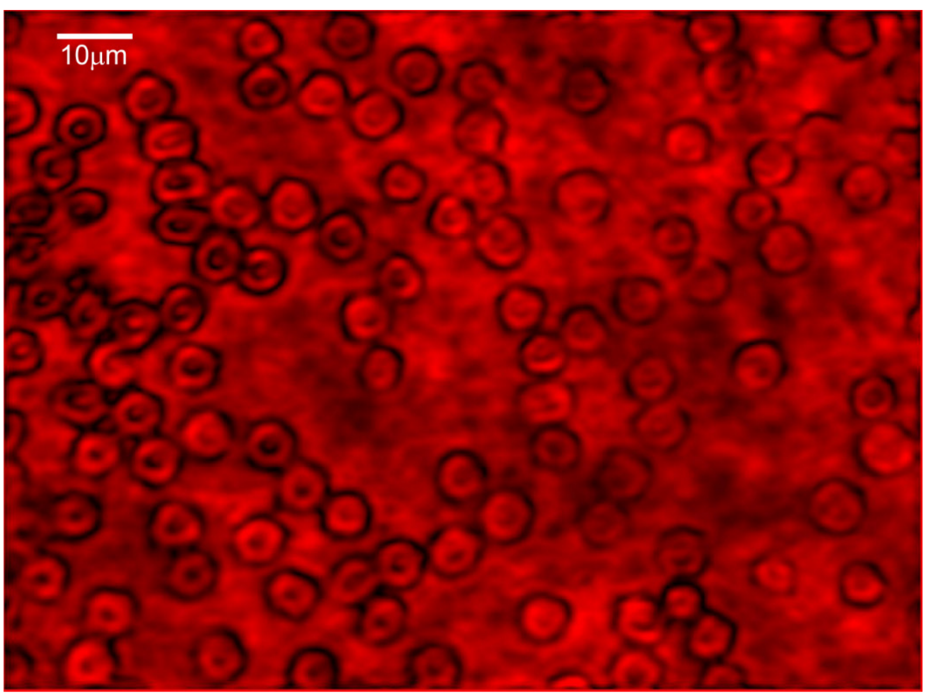

Fig. 1.1: Bright field microscope image of human red blood cells. Magnification was achieved using a positive lens of 6mm focal length.

Image contrast in bright field microscopy is caused by different response (absorption/reflection) of sample and background to the incident light. For high contrast imaging, the absorption/reflection by/from the sample should be much higher than the background. So bright field microscopy is not preferred for semi-transparent specimens as (like cells), as they do not produce visible change to amplitude of incident radiation and hence yields poor contrast. This is usually improved by treating the sample with chemicals. Dark-field microscopy technique can also be used for achieving contrast between object and the surrounding medium [3-4]. As the name suggests, in dark field microscopy, the object under observation is bright and the background is dark. It is usually achieved by oblique illumination of the object under observation. The main disadvantage of dark-field microscopy is that the light scattered by the specimen is less resulting in low flux at the image plane. But even with its disadvantage of low contrast images, bright field microscopes are most commonly used for imaging and identification of cells and micro-organisms mostly after staining the sample. A bright field system employing a single lens for magnification has many advantages over the existing compound microscopes which uses a relay of lenses. One of the advantages is that the microscope can be constructed with few optical elements, which reduces its cost. Also, since few optical elements rea required, it can be made very compact, making hand-held field portable microscopes a reality. By using multiple illuminating wavelengths, the contrast as well as information content can be improved.

Chapter 2 presents, the design, development and fabrication of hand-held bright field microscopes. The devices were fabricated using DVD pick-up lenses for magnification of the object and LEDs working at different wavelengths for sample illumination. A low-cost webcam was used for recording the images. The webcam was connected to a smart-phone, which acted as the image acquisition and storage device. Use of smart-phone also makes it possible to use the device for point-of-care applications. The recorded intensity patterns were sent to an off-site computer via internet. Simple image processing algorithms based on thresholding the images with gray values were used to classify the cells [9-10]. *The images were processed (using in-house developed software) in the computer and the obtained sample parameters were send back to the user. The device was used for imaging and automatic identification of stained blood cells.*

1.3 Two-beam quantitative phase microscopy

As mentioned in the previous section bright field microscopes suffers from the lack of contrast especially in the case of unstained biological samples. Even with staining the thickness information (and hence object morphology) is not available using bright field microscopes. Fluorescence microscopy overcomes the limitation of bright-field microscopy by improving contrast between the structures in the cell, as well as providing information on the thickness/depth of the sample [5]. A fluorescence microscope is similar to conventional bright field microscope with additional features. In this imaging modality, the sample is tagged/labelled with fluorescent dye (fluorophores), which gets attached to specific portions of the sample. This is then illuminated with the excitation (absorption) wavelength corresponding the dye [5]. The excited fluorophores emits a longer wavelength. A filter selects allows only the emitted longer wavelength to pass through, while blocking the excitation wavelength. So a fluorescent image brings out only area of interest which is tagged with the fluorophore and hence also contains depth information. The contrast and sectional properties of fluorescent imaging can be improved by the use of confocal microscopy [6]. This is achieved by letting in only the light from the point of interest (blocking all other light) by the use of a pin hole at the detector plane. It is improves the sectioning as well as contrast properties of the microscope. Every point in the specimen is scanned by either moving the beam or the sample, allowing reconstruction of 2D image at a given plane. Another advantage of confocal microscopy is that one can move the specimen vertically and images at different depths can be obtained. The 3D image can be reconstructed with the aid of proper software. But fluorescent microscopy and confocal microscopy increases the complexity and bulkiness of the device. Also trained technicians are required for labelling/tagging the sample, data acquisition (which involves a scanning) and for data analysis. Due to scanning process, these imaging modalities are not single shot in nature. To obtain information about the whole sample requires multiple images of the sample and is time consuming.

Another way to improve contrast is to detect the phase of the light interacting with the sample (object beam) rather than recording its intensity. This leads to phase contrast imaging [3-4, 7, 11-12]. Optical This is usually achieved by superposing object beam with a known reference beam, leading to creation of interference fringes [13]. These interference fringes contain information about the phase of the object beam in the form of the modulation of the interference fringes [7].

By processing these fringes, the object phase information can be extracted [7]. The phase information is related to the object optical thickness and hence the cell morphology (thickness variation) can be reconstructed from the interference pattern leading to quantitative (thickness) phase imaging. Digital holographic interference microscopy (DHIM) [14-108] is the state of the art quantitative phase imaging modality having the added advantage of numerical focusing [18-108]. In DHIM, the interference pattern (this interference pattern can be considered as a *hologram* since it contains both amplitude and phase information) between the object beam and reference beam are recorded on digital arrays (CCD/CMOS) as shown in Fig. 3.10 (detailed explanation is given in Section 3.3). This pattern is processed by numerical implementation of diffraction integrals [18, 101-106] to yield the object phase information at the best focus plane. It should be noted that the focusing is achieved post-recording of the hologram [101-105, 108]. This phase information contains the object thickness information [18]. Fig. 1.2 [90] shows the phase images of the same set of cells shown in Fig. 1.1. The improvement in contrast is quite evident.

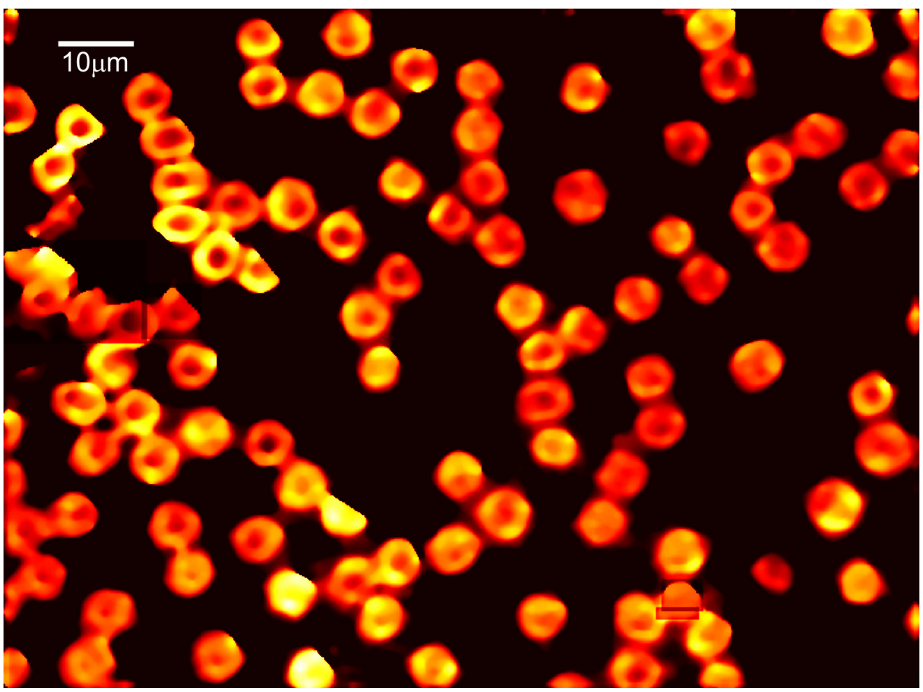

Fig. 1.2: Quantitative phase image of blood cells obtained using wavefront division DHIM [90].

Reconstruction of the object information (amplitude and phase) from a single hologram, also known as single shot reconstruction, require an off-axis superposition of object and reference beams [109] as shown in Fig. 3.10. This require the use of a two-beam configuration in which a separate object beam and a reference beam, which are derived from the same source, travel along different paths and interfere at the recording sensor [13, 19, 23-26, 29-30, 32-76]. Also since most microscope investigations are carried out in the transmission mode, the most widely used interferometer geometry for construction of transmission mode DHIM is the Mach-Zehnder configuration [13, 19, 23-26, 29-30, 36-76]. This configuration ensures, same path lengths between the object and the reference beams leading to high contrast interference fringes. This configuration can be used to produce high quality thickness profiles of cells as well as technical objects and to measure the refractive index distributions inside them [38, 40-42, 46-49, 51, 61, 63-66, 70, 72-73] and can be used to monitor their dynamics [50, 52-53, 55-50, 67, 71, 74, 75]. Several parameters based on the cell morphology and its time variation (including cell thickness, cell volume, cell surface area, cell sphericity, cell refractive index, cell thickness fluctuation amplitude, cell thickness fluctuation frequency) can be extracted from the recorded holograms (or a time series of holograms) using this configuration [38, 40-42, 46-47, 49-52, 55, 57-58, 60-61, 63-68, 70-76]. These parameters along with other information obtained from phase profile provides insight on the state of existence (including health) of the cells and have been used to characterize and classify cells [23-27, 29-32, 42, 47, 56, 58-59, 61, 63-68, 70-76].

Chapter 3 details the design and construction of two beam digital holographic microscopes employing Mach-Zehnder interferometer configuration. It utilized He-Ne laser sources as well as laser diode modules along with CCD arrays. The developed microscope was used for quantitative phase imaging of blood cells. Thickness profile of blood cells were reconstructed from the phase images and cell parameters based on cell morphology were extracted. These parameters were used along with statistical algorithms for characterization, identification and classification of healthy and malaria tested positive blood samples [63, 72-73]. *The technique was found to yield high quality phase reconstructions of blood cells. Also the technique was able to classify healthy and malaria infected red blood cells automatically.*

1.4 Self-referencing off axis DHIM using lateral shearing geometry

Two-beam DHIM based on Mach-Zehnder configuration requires, optical components for beam splitting, beam steering and beam superposition. This leads to bulky setups, which may hinder its on-field applications. Dynamic imaging of cells (especially red blood cells) require measurement of thickness variation of the order of few 10s of nanometer over extended period of time [78]. In two-beam geometry, the object and reference beams travel along two different paths which may result in them picking up uncorrelated path length variations leading to lower temporal phase stability (2-3nm over few minutes), which make it not so suitable for measurement of the cell thickness fluctuations without vibration isolation mechanism. A geometry in which the object and reference beams travel along the same path and possibly coming into contact with the same optical elements will lead higher temporal stabilities [76-99]. Usually in such common path setups, the reference beam is derived from the object beam (after it passes through the magnifying lens). There are two options in such a geometry. In the first one, two versions of the object beam are created (using a beamspliter, grating etc). A separate reference beam is then fashioned out from one of these beams by eliminating object information from it, usually by spatial filtering, using a lens and a pin-hole [77-86] or just by using a pin-hole in the path of the object beam [87]. Separate reference beam can also be created by splitting the beam illuminating the sample before magnification lens using a grating also [88]. Method using two-lenses to create a separate reference beam can also be employed [89]. But the simplest method is the one which uses a clean portion (not containing object information) of the object beam as the reference and superposing this with the portions of the same wavefront having cell information [90-99]. This leads to the area of self-referencing DHIM. This technique is simple an can be used to create microscope geometries which uses very few optical elements and are very compact, which makes it possible to convert them into portable quantitative phase microscopes. One of the easiest self-referencing geometry uses a glass plate to generate two laterally sheared versions of the object wavefront [17, 76, 93, 95-96]. These two wavefronts interfere at the sensor plane. Clean regions of the waterfront (either from front or back surface of the glass plate) act as the reference. Since the technique involves the use of very few optical elements and since the object and reference beams travel along the same path and interact with the same optical elements, such a setup can provide sub-nanometer level temporal stability [93].

Chapter 4, details the development of field portable, low cost lateral shearing DHIM. Compact devices were fabricated by using off the shelf optical components. The magnifying lens was fashioned out of a DVD optical pick-up unit, which allowed image focusing by applying a current to the voice coil of the pick-up unit. Also multiple filed of views were achievable by lateral translation of the pick-up lens, again by applying a current to the voice coil. As the digital array was at the image plane of the magnification lens, a simple Fourier analysis of the interference fringes [109] *led real time quantitative phase imaging of cells. The developed device could be* *attached to smart-phones which acted as hologram recording and transmission device. In the point-of-care devices, the recorded holograms were transmitted to an off-site computer, in which the in-house built computer programs, reconstructed holograms and extracted the cell phase profile and cell parameters. These parameters were then used for sending an educated guess on the state of health of the cell back to the user.*

1.5 Two-lens wavefront division DHIM

Lateral shearing DHIM provides a compact setup, but it requires sparse object distributions so that a portion of the object beam is clean from object information [93]. Also the reference portion of the object beam may also contain object background information. This may lead to unwanted spatial phase fluctuations (which ideally should be zero) resulting in lower spatial phase stability. A setup which provides the imaging quality of Mach-Zehnder configuration (due to separate totally clean reference beam) while keeping the common path nature and compactness of the lateral shearing geometry is desirable.

Separate reference beam is usually achieved by spatially filtering and erasing object information from one of the two versions of the object beam and combining it with the beam containing object information [76-87]. This requires optical components for spatial filtering (lenses and pin-hole) and beam ratio adjustment for high contrast interference fringes. An alternate technique is to employ wavefront division DHIM geometry [88-89, 94, 96]. It can be used either in self-referencing mode [94, 96] or to generate a separate reference beam [88-89].

In the wavefront division geometry with a separate reference beam, the laser beam (used to illuminate the object) is split into two before it illuminates the object [88-89]. In one of the work reported in literature, a grating engraved on the microscope slide, created a separate reference beam from a portion of the laser beam illuminating the microscope slide. The other portion of the illuminating beam passes through the object, which was in a microfluidic chamber [88]. A simpler way is to allow half of the illuminating beam to pass through the object and then through the magnifying lens and the other half to pass unobstructed through another lens of the same configuration (same focal length and clear aperture) [89]. This beam will act as a separate reference. Also since the two lenses are of the same configuration, automatic matching of the wavefront curvatures as well as intensities occur which leads to high contrast linear fringes.

Chapter 5, provides details of the developed two-lens wavefront division DHIM. It used a wavefront division quantitative phase imaging module comprising of two lenses placed close to each other. Object was placed behind one of the lenses. A portion of the illuminating beam passed through the sample and was magnified by this lens. The other portion of the same wavefront, which is clean was allowed to pass through the second lens and acted as the reference beam. The two beams were made to interfere at the sensor plane. The spatial separation between the lenses, create an angle between leading to off-axis geometry. The method is easy to implement and can be constructed just with the wavefront division module making it compact. It was tested by measuring the physical and mechanical parameters of blood cells.

1.6 Integration of low cost optical tweezers to self-referencing DHIM

The methods described so far uses, samples fixed on microscope slides for quantitative phase imaging. But it is ideal to study cells in their natural environment, which is in fluids. But in fluids the cells are not fixed to the same position and it becomes difficult to measure properties of single cells. A way to immobilize cells in their natural environment is to use radiation pressure by illuminating the cells with a tightly focused laser beam [110-113]. It finds wide range of applications in the areas of physics to biology for immobilizing and moving objects of size ranging from single molecule to 100s of micrometers [114-126]. It has found application in study of stem cells [114-115], tumor cells [116], viruses and bacteria [117], discrimination of healthy and diseased cells [118], genetic testing [119], single DNA mechanics [120], cell biosensors [121], measurement of elasticity of cells and optical cell sorting [122-125], and measurement of oscillation frequency of cells [126].

The ability of the optical tweezer to fix the cells to a particular position makes it an ideal tool to observe and quantify cells in its natural environment. If a DHIM device can be integrated with an optical tweezer, it will provide the user with additional information on the cell, which will depends upon the state of health of the cell. This will prove to be boon for cell characterization, identification, and classification and sorting. But the cost of commercially available optical tweezers run into ten thousands of USD since it requires high power laser sources, Quadrant photo-diodes (QPD), imaging systems etc. But optical tweezers could be realized with low cost laser sources and lenses available in DVD optical pick-up units, which can provide laser powers up to 250mW as well as short focal length lenses with high numerical aperture required for tight focusing of the laser beam [127]. This when coupled with off the shelf imaging lenses and imaging unit can lead to low cost optical traps [127].

As described in Section 1.3, lateral shearing DHIM is a compact version of two-beam DHIM. Can the low cost optical tweezers based on DVD optical pickup be integrated with lateral shearing DHIM is the problem addressed in Chapter 6. The optical tweezer was realized using DVD optical pick-up unit and low cost magnification lenses and imaging sensors. A low cost QPD unit was fabricated using four large area photodiodes and optical fibers. This was used for corner frequency measurement of trapped objects. A DHIM unit comprising of a glass plate and a webcam was introduced into the trapping setup for quantitative phase imaging of trapped objects.

1.7 Outline of the thesis

The work described in this thesis focuses on development, fabrication and application of compact, field portable, low-cost bright field and DHIM devices for cell classification, quantitative phase contrast imaging and quantification of micro-objects, especially biological cells. Thesis describes the development of various compact microscopes based on different geometries especially in the case of DHIM devices. Chapter 1 provides an introduction and overview of the microscopic imaging. Progression from bright field imaging to quantitative phase imaging is also provided in this chapter. This chapter also introduces the concept of common path DHIM techniques and explain their difference from two-beam techniques. Chapter 2 is concerned with the development of compact bright field microscopes using off the shelf low cost optical and imaging components. It also explains the image processing steps involved in cell identification leading to cell counting and disease diagnosis. Chapter 3 introduces the two-beam DHIM employing Mach-Zehnder interferometer geometry. Application of the two-beam DHIM in automatic identification of malaria infected blood samples is also provides. Chapter 4 introduces the concept of compact common path self-referencing geometry using lateral shearing interferometer for quantitative phase contrast imaging. It also provides explanation on the design and fabrication of field portable version of the DHIM device that could be attached to smart phones. Chapter 5 explain the development of wavefront division DHIM using a two-lens system and its application in cell imaging. Chapter 6 describes the integration of low-cost optical tweezers with lateral shearing DHIM. Finally Chapter 7 summarizes the efforts made for development of compact microscopes and provides a short discussion on the future directions.