DESIGN, INSTRUMENTATION AND FABRICATION OF COMPACT OPTICAL MICROSCOPES FOR CELL IMAGING AND ANALYSIS

A Synopsis of the Thesis Submitted to the

THE MAHARAJA SAYAJIRAO UNIVERSITY OF BARODA

In partial fulfilment of the requirements
for the award of Degree of

DOCTORATE OF PHILOSOPHY

In

APPLIED PHYSICS

by

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CHAPTER 1

Introduction

Every year, millions of deaths occur in developing and under developing countries due to lack of diagnostic facilities and remote testing tools. In 2016, 216 million people suffered from malaria in which 445000 people died which constitute 70% of children under the age of five [1]. One-third of the world population is infected with tuberculosis and in 2016, 10.4 million people got sick with the fatal disease and around 1.7 million deaths are reported [2]. African trypanosomiasis is a disease which affects 10,000 people every year and is reported to WHO, however many cases go unnoticed and proves to be fatal if left untreated.

Today, Bright-field Microscopy still stands as a gold standard technique for diagnosis of many diseases and illness in hospitals and clinics as well as primary inspection method for microscopic samples. 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 easily. For semi-transparent microscopic objects, it is necessary to have good contrast between the foreground and background. Based on contrast enhancing principle, many microscope techniques are available to increase the specimen contrast such as fluorescence microscopy, confocal microscopy, iterative phase retrieval techniques etc. In Bright field microscopy [3], the light from the source is either passed through, or reflected off, a specimen. Contrast in the specimen is caused by different amplitude response of sample and background to the incident light. Bright field microscopy is not preferred for semi-transparent specimens as, they do not produce visible change to amplitude of incident radiation and yields poor contrast, specificity and sensitivity. To increase the contrast samples can be stained using dyes with high absorption coefficient. But staining of sample may introduce inessential details into the cells that should not be present and may kill the living cells.

Fluorescence microscopy overcomes the limitation in bright-field microscopy by improving contrast between the structures in the cell, as well as allowing you to collect images in more than one colour. A fluorescence microscope is similar to conventional bright field microscope with additional features. In fluorescence microscopy, the sample of interest is tagged with fluorescent dye (fluorophores) and then illuminated with the light of specific shorter wavelength (higher energy) source [4]. The microscope has an excitation filter which lets through radiation of specific wavelength depending on the fluorophore used. The illuminated light is absorbed by the fluorophores (attached to the specimen) and cause them to emit longer wavelength (lower energy) light [5]. The illumination light is blocked by the emission filter which allows only weaker fluorescence to pass. Since the emitted light differs in wavelength(colour) from the excitation light, an ideal fluorescent image shows only area of interest which was labelled with the fluorophore.

The low contrast limit in the bright-field microscopy can also be overcome by phase contrast microscopy which has ability of converting phase change into amplitude change. The application of phase contrast

microscopy would make objects appear brighter or darker, thereby increasing their colour contrast with the surrounding medium. Phase contrast microscopy utilises the fact that the light passing through the portion of specimen travels slower which creates phase shifts when compared to the phase of light passing through background [6]. This difference in the phase is very difficult to detect with normal bright field microscope without staining the sample. However, this phase change can be increased to half a wavelength by employing transparent phase plate in the microscope [7]. Thus, this technique converts phase shifts caused by the specimen to brightness change in image. As a result, semi-transparent biological living cells can be imaged with high contrast and clarity of minute details in specimen, which is impossible to image without staining using bright field microscopes. This technique is not ideal for thick specimen or cells as thick specimens can appear distorted. Bright field microscopy provides only two-dimensional (2D) intensity information at imaging plane. It lacks specimen height/thickness information. This limitation can be overcome by employing quantitative phase microscopic (QPM) techniques. Interferometric QPM techniques are widely used for obtaining cell morphological information as well as variation in its morphology with time. Digital holographic microscopy (DHM) is one of the state of-the-art methods for quantitative three-dimensional (3D) imaging [8]. The commonly implemented two beam (Mach-Zehnder) setup is prone to mechanical vibrations and produces low temporal stability which is not desired in cell dynamics study [9]. one needs temporal stability much better than the fluctuations of the object, which the two-beam geometry fails to deliver. To overcome this barrier, one can use self-referencing techniques, in which a portion of the object beam will act as the reference beam.

To study microscopic cells in its own surrounding medium, one has to manipulate and immobilized the cell in 3-dimension. To achieve this, optical tweezers can be utilized [10]. Optical tweezers can be used to trap 10 to 10,000 nanometer sized particles such as dielectric polystyrene spheres, bacteria, living cells, organelles, small metal particles, and even strands of DNA [11]. Applications of optical tweezer include cell sorting [12], tracking of movement of bacteria [13] and altering of larger structures (such as cell membranes). Incorporating LSDHM with optical tweezer, one can study static and mechanical parameters of the trapped particle.

Microscopes used by pathological labs are not designed for field testing and remote diagnosis. A Point-of-care diagnostic tool is desired where access to the health network is challenging, which can provide low-cost medical tests and doesn't required trained personnel. In this work, Compact, low-cost, 3d printed microscopes such as bright-field microscope, Fluorescence microscope, microscopes based on Quantitative phase contrast (3D imaging) techniques and optical trapping device are developed. A web based interface was developed so that the data can be send to server over internet and the computed results are send back to user.

CHAPTER 2

2.1 Compact, Low-Cost, 3d Printed Bright-Field Microscope

A traditional compound microscope is too bulky for remote field applications. We have developed a compact, lightweight bright field microscope with minimum components and yields similar results compare to traditional microscope.

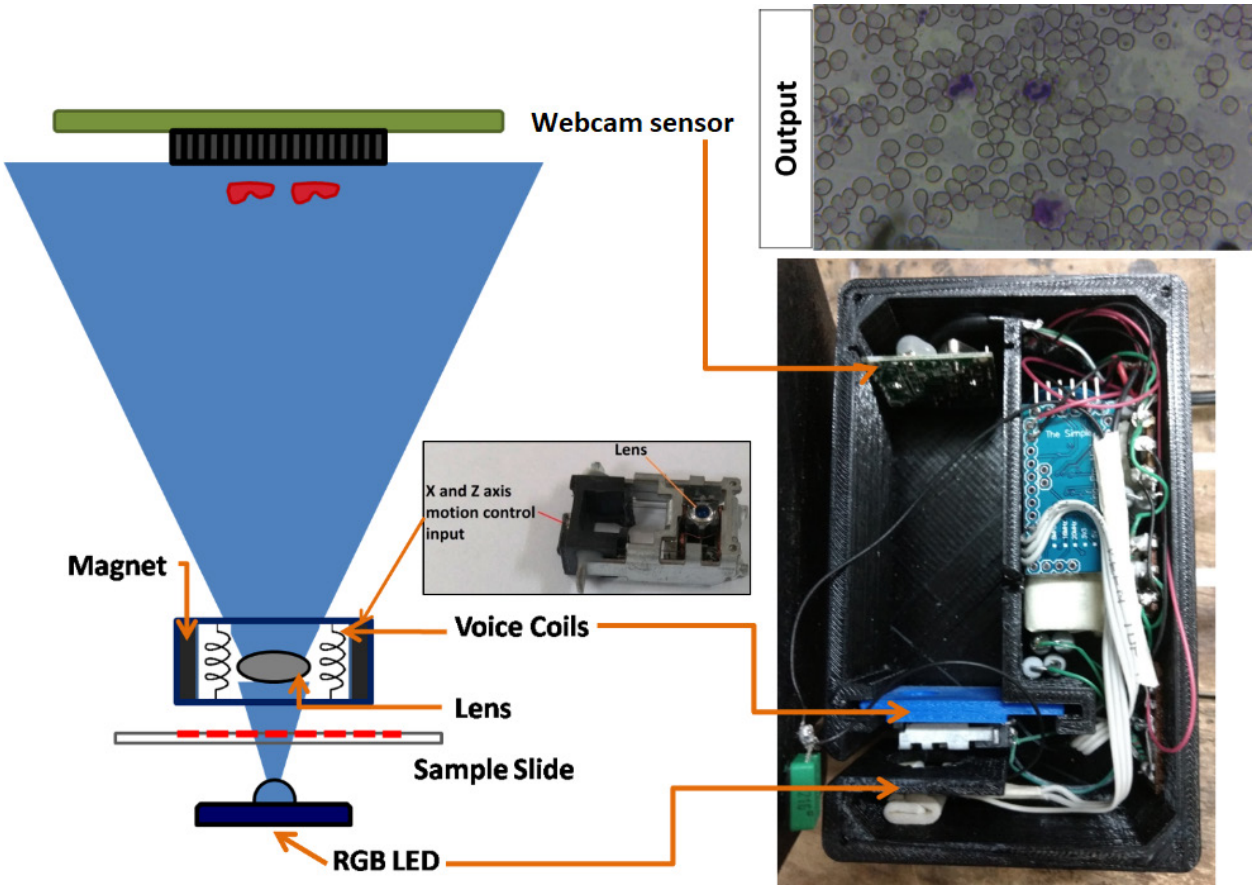


Figure 2.1.1 Working principle and inside-view of developed BFM

The developed bright field microscope consists of a RGB led used for the illumination of sample. The magnification and focusing of the sample is carried out by electro-mechanically moving lens extracted from the DVD optical pickup [14, 15]. The lens used in has numerical aperture of 0.6 and having focal length 2mm. The lens rests between two permanent magnets on a platform having two voice coils which controls the position of lens in Z-axis and X-axis when varying current passed through them. A separate indigenous control unit was developed for controlling amount and direction of current with the aid of potentiometers causes 1D translation motion and focusing movement of the magnifying lens. The magnified image is recorded by the webcam sensor which is attached to PC or Android device. Application of RGB led serves selection of source colour, which provides better contrast images while observing specific stained specimens. The translation motion of the lens renders ability to scan over the specimens and large Field of view can be generated by combining all the captured images. The Compact bright-field microscope developed here serves more advantage in terms of providing point of care diagnosis. Table 2.1.1 highlights the comparison between the traditional microscope and the developed one.

Table 2.1.1 Comparison between Traditional Compound Microscope and Developed BFM	
Traditional Compound Microscope	**Developed compact BFM**
It has many moving components.	Only Electro-mechanically moving lens.
It's a 3-lens system.	Single lens system.
bulky in size, not field portable.	Compact in size (7 x 4.5 x 11.5) cm3
Continuously use causes eye strain.	No eyepiece incorporated. Images recorded via Sensor to PC/Phone.
Magnification can be varied (10x, 40x, 100x etc.)	Magnification is fixed (40x), separate device for different magnification.
Required external power source.	Fully battery powered.

2.2 Compact Low-Cost Fluorescence Microscope (Converted from Developed BFM)

The developed compact, bright-field microscope can be converted to Fluorescence microscope simply by addition of two filters. We have developed Fluorescence microscope, specially designed for malaria diagnosis based on Kawamoto technique [15, 16-18] in which excitation light (470-490nm) is allowed to passed through Acridine Orange stained specimen [19, 20]. The second filter (510nm) is placed before the imaging sensor which allows only fluorescence from the infected cells to pass. One has to note that Acridine Orange is very intense fluorescent dye, and it stains nucleic acids from all cell types. Consequently, one must learn to distinguish fluorescence tagged parasites from other cells and cellular debris.

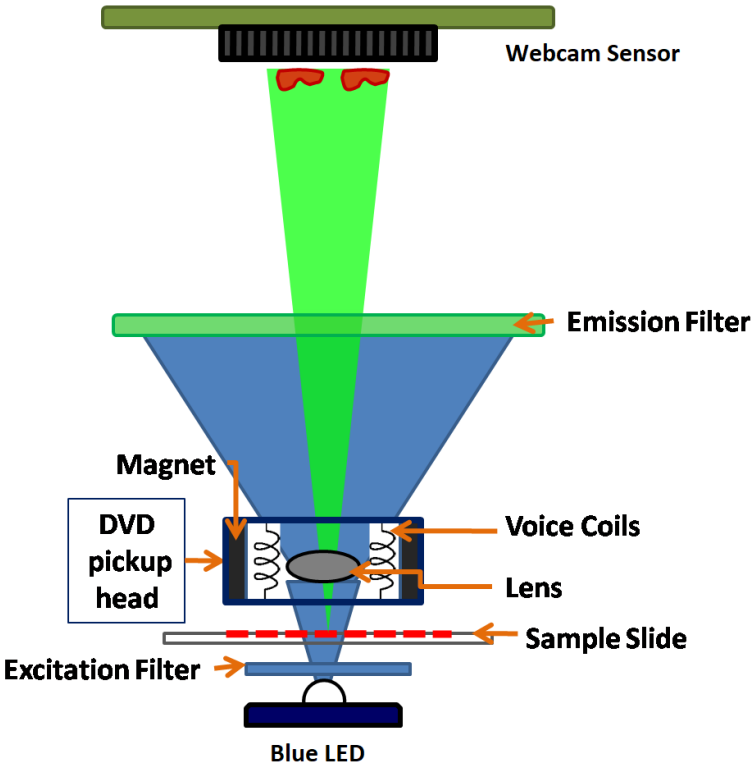


Figure 2.2.1 Schematic of developed Compact Fluorescence microscope

As depicted in figure (2.2.1), setup is equipped with 2-watt blue led source with excitation filter which allows light within 400nm to 480nm wavelength range. As the same bright field microscope discussed in the above section is used for fluorescence microscope, the necessary changes like grooves are designed in that original BFM version to insert the excitation and emission filters keeping the magnification, focusing and imaging system same. Emission filter of central wavelength 510nm having bandwidth of 10nm is used before webcam sensor to record fluorescence images.

CHAPTER 3

Digital Holographic Quantitative Phase-Contrast Microscopy

Quantitative phase-contrast microscopy (QPCM) is collective name of microscopy techniques such as digital holographic microscopy, holographic interference microscopy and digital in-line holographic microscopy that quantify the phase shift that occurs when light waves pass through transparent specimen or a more optically dense object [21-26]. QPCM provides many advanced imaging techniques that offers real-time imaging of phase objects such as micron size polystyrene beads and quantitative measurements of physiological parameters of biological cells [27-34]. It allows us to study living cells without staining or labelling them and without use of chemicals which may affect their properties them. Digital holography microscopy makes it possible to easily measure cell properties such as Cell thickness, Surface area, volume, fluctuations, refractive index, motility etc. [35-38], which lacks in Bright-field microscopy. Our aim is to demonstrate Quantitative phase-contrast microscopes as haematology analysers.

3.1 Identification of Malaria and Healthy Cells using Digital Holographic Microscope (Mach-Zehnder Configuration)

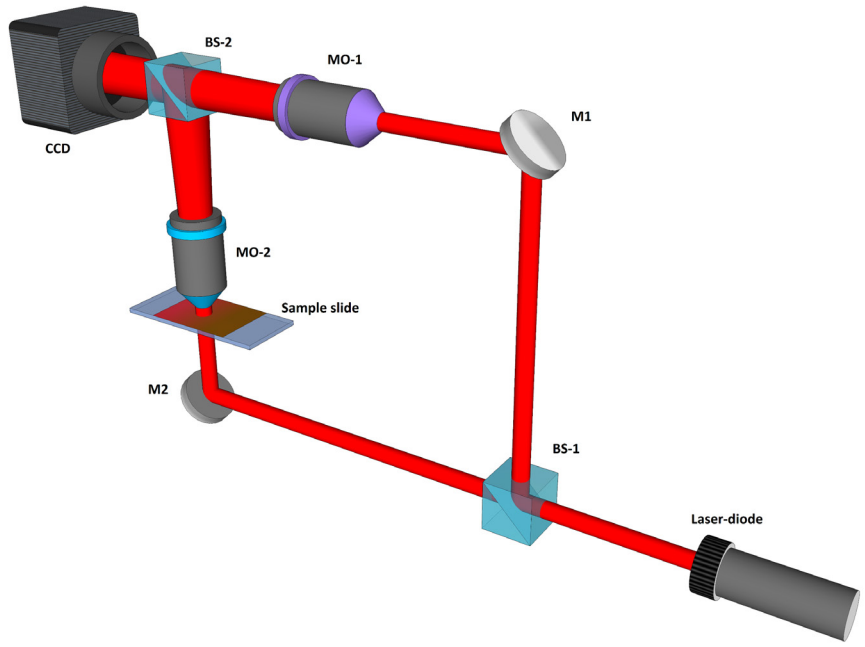


Figure 3.1.1 Schematic of the digital holographic microscope using Mach-Zehnder configuration

Schematic of the digital holographic microscope using Mach-Zehnder configuration is as shown in figure. (3.1.1). In Mach-Zehnder Configuration, the expanded and collimated laser beam was split into two (Amplitude Division) using a nonpolarizing beam splitter (using BS-1 and BS-2). One of the beams acts as the reference beam, and the other beam which passes through the object under investigation, acts as the object beam. A microscopic objective (MO-2) magnifies the object. Object beam is then magnified and then made to interfere with the reference beam at the sensor plane called as hologram. A similar kind of microscopic objective (MO-1) is introduced in the reference beam path to match the curvatures of the beams, and a slight angle is introduced between the object and reference beams to achieve off-axis geometry [21,22]. The resulting interference patterns (holograms) were recorded using CCD/CMOS sensor and stored in PC for numerical reconstructions which were carried out using angular spectrum propagation (ASP) integral [27].

The biological application of this setup is discussed by discrimination of malarial and healthy red blood cells based on statistical static properties like diameter of cells, surface area, curved surface area, optical volume, thickness, projected area, coefficient of variation of thickness and optical volume. The correlation between different parameters is also used for identification.

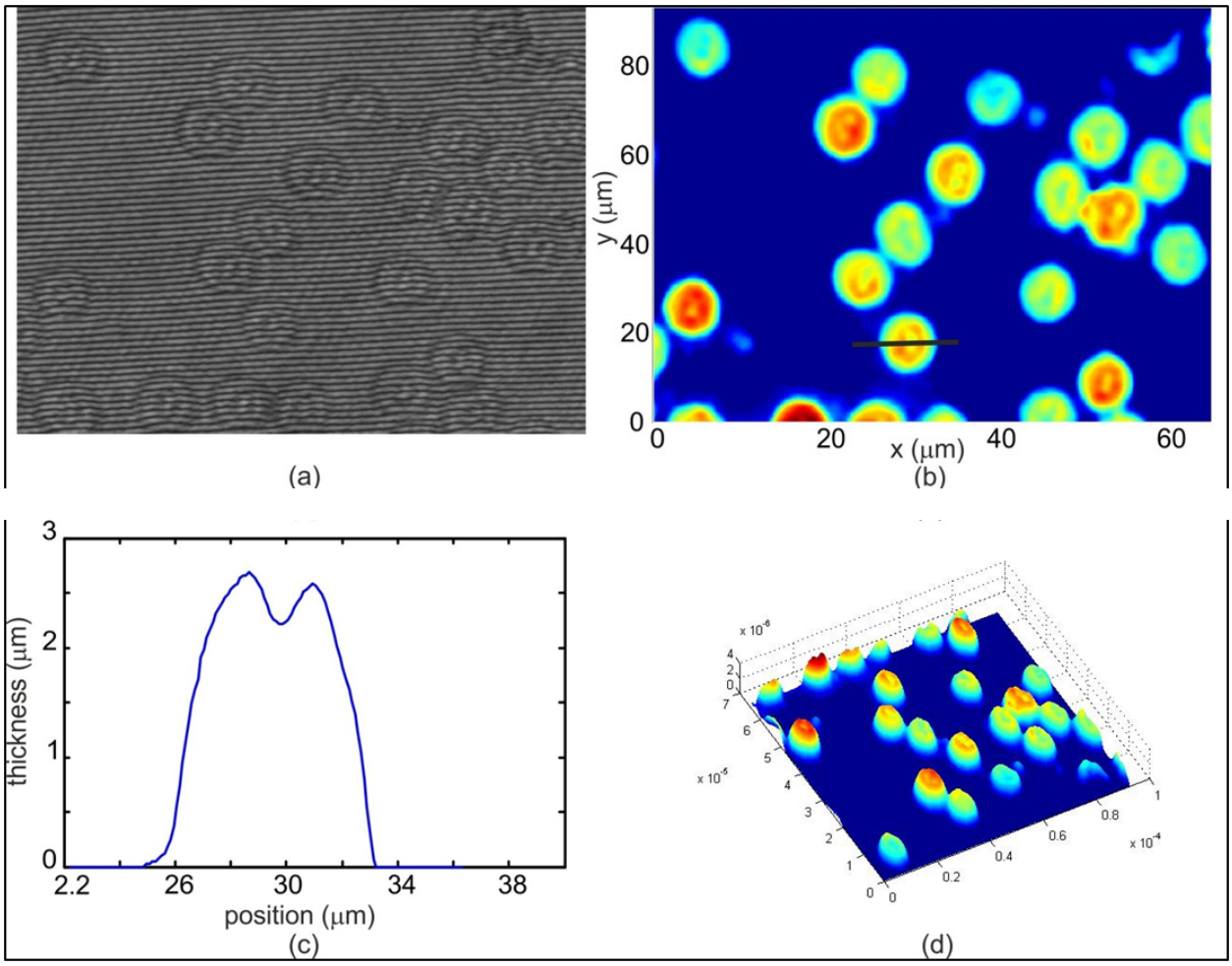


Figure 3.1.2 (a) Object Hologram, (b) Phase distribution, (c) 2D Line profile and (d) 3D profile

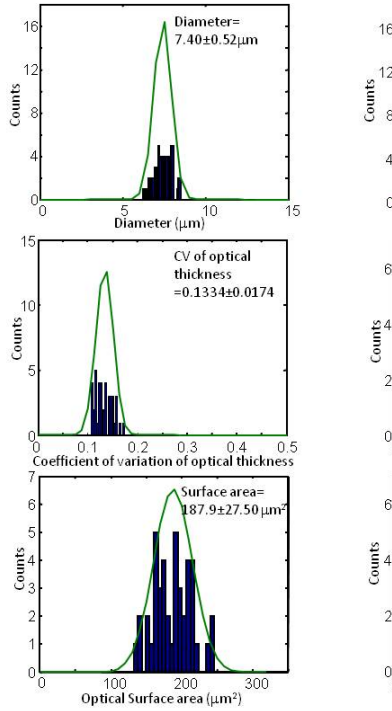


Figure 3.1.3(a) Distribution plots (Healthy RBCs)

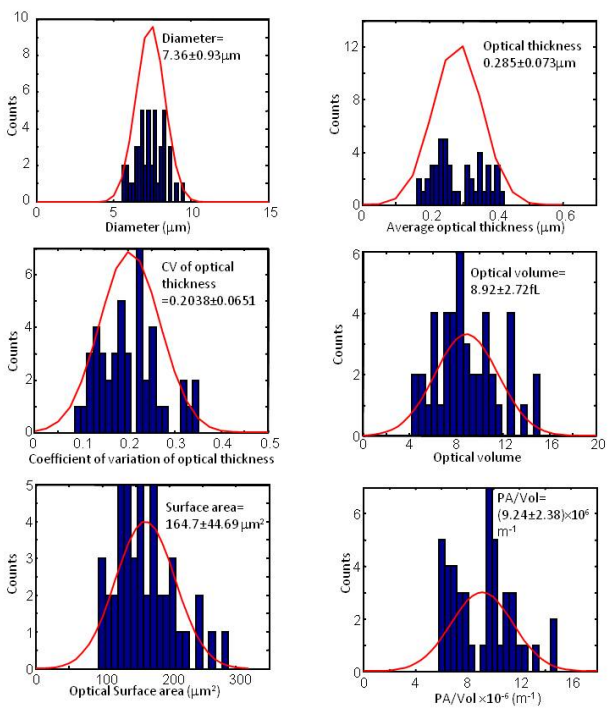


Figure 3.1.3(b) Distribution plots (Malaria Infected RBCs)

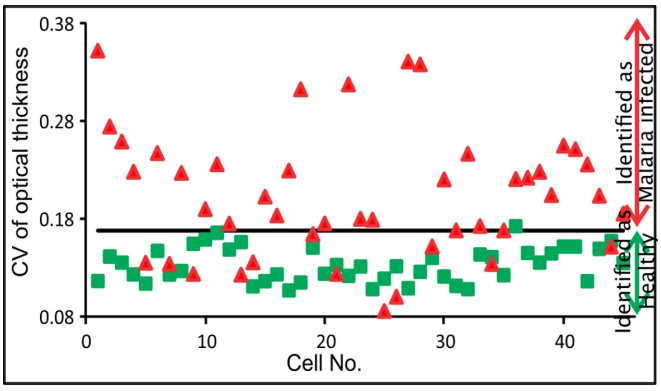


Figure 3.1.3(c) Identification of Malaria using CV of Optical thickness

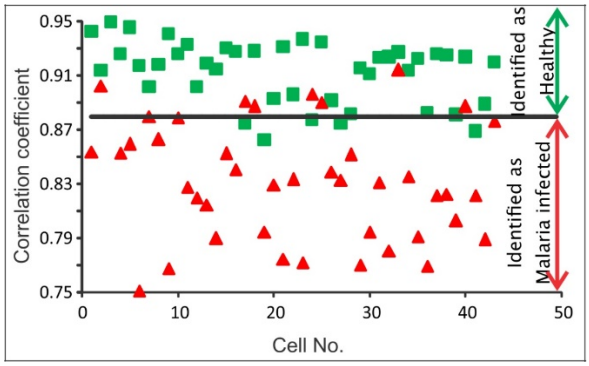


Figure 3.1.3(d) Identification of malaria using Shape correlation

CHAPTER 4

4.1 Digital Holographic Microscope Using Shearing Glass Plate (Version-1)

In lateral shearing digital holographic microscope (LSHDM) [39-45], The laser beam is directed towards the specimen and subsequently magnified using microscopic objective. The shearing of the beams is achieved by a thick glass plate oriented at 45° to the microscopic objective. Interference occurs between

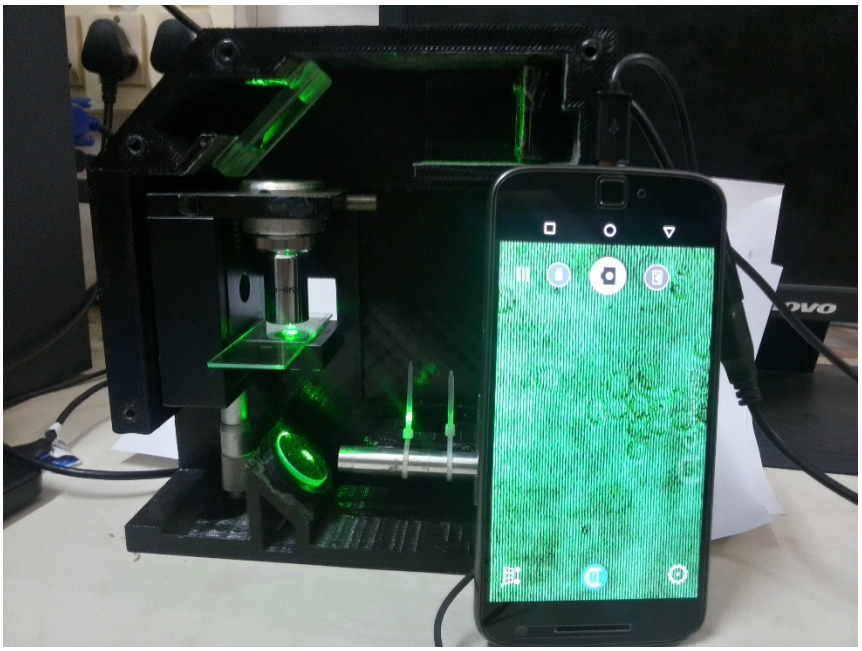


Figure 4.1.1 Developed 3D printed LSDHM Device

a wavefront coming from the front surface of the glass plate and the shifted and duplicated wavefront of the same beam reflected from the back surface of the glass plate. Both the front and back reflected beams carry specimen information. So, unperturbed region of one beam acts as a reference for the other beams carrying specimen information for the same region. Limitation of this technique is one gets two images of the same object on sensor FOV. Of course, it is possible to image only one object by increasing amount of shear between the beams.

Results…

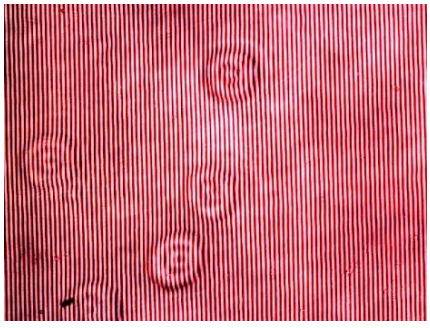
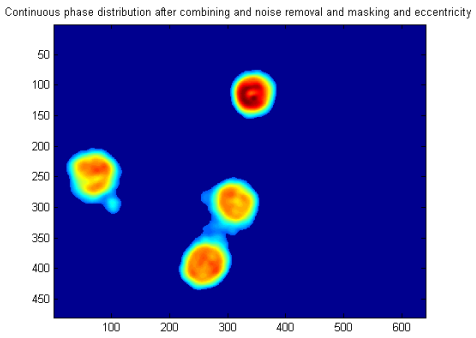


Figure 4.1.2(a) Object Hologram



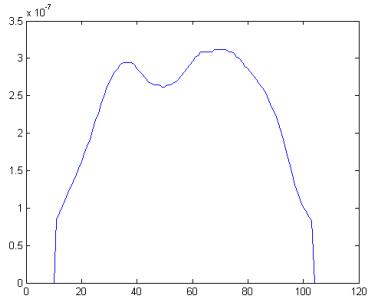


Figure 4.1.2(c) 2D Line profile

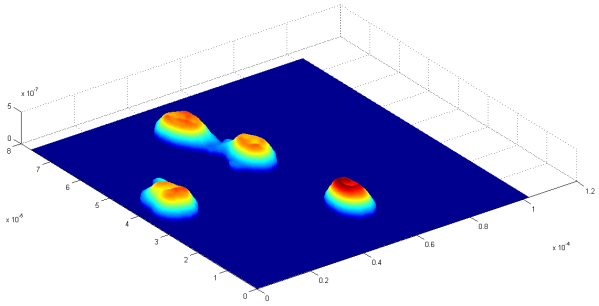


Figure 4.1.2(d) 3D profile

4.2 Compact Lateral Shearing Digital Holographic Microscope (Version-2)

Utilization of standard microscopic objective and translation stage in first version of LSDHM, the developed device gets a bit bulky and unfavourable for remote field applications. In this work, compact pocket size holographic microscope for remote field diagnosis was developed, the standard objective lens was replaced by aspheric singlet objective lens (FL = 2mm, N.A. = 0.65) salvaged from the DVD drive pickup head [46, 47]. The DVD pickup lens rests on a platform having two voice coils which moves the lens upon passing the current through them. From the mean position, objective lens can be steered in both X and Z direction [48, 49] with the precision of fraction of a micron. Application of DVD pickup lens greatly reduces the size of the device and yields a handheld Holographic microscope that offers real time imaging and quantitative measurement of physiological parameters of biological cells [50, 51]. A web-based interface for automated cell parameter extraction [52] has also been developed for remote analysis of the sample. Recorded holograms can be send over web-interface to remote server for cell parameters extraction and computed results can be send back to user.

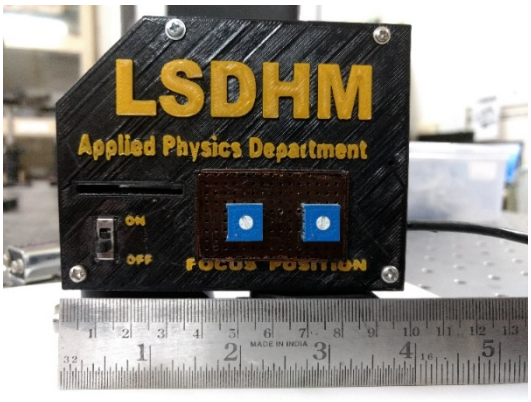


Figure 4.2.1 Developed 3D printed compact LSDHM

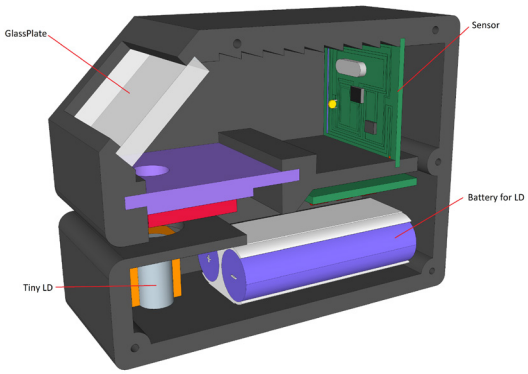


Figure 4.2.2 Inside view of Compact LSDHM

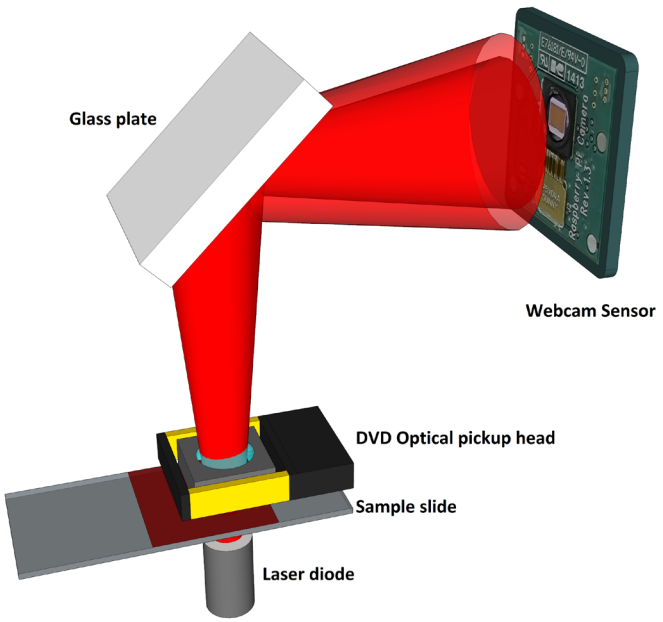


Figure 4.2.3 Schematic of Compact 3D LSDHM

Results…

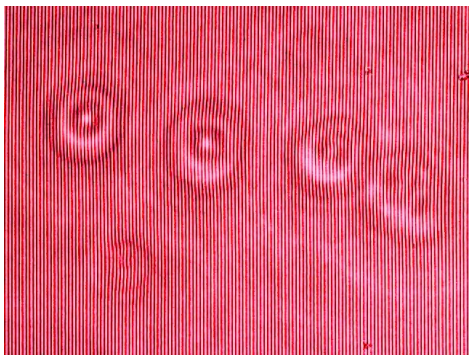


Figure 4.2.4(a) Object Hologram

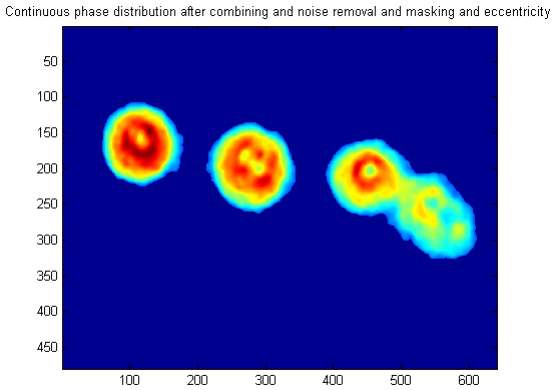


Figure 4.2.4(b) Phase Distribution

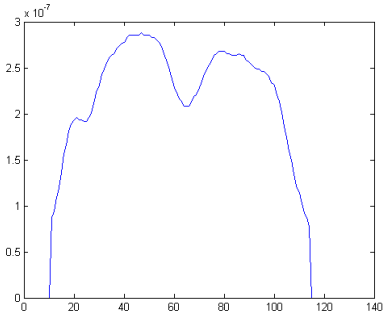


Figure 4.2.4(c) 2D Line profile

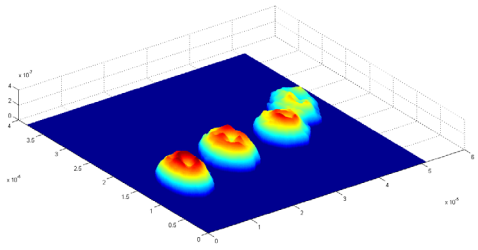


Figure 4.2.4(d) 3D profile

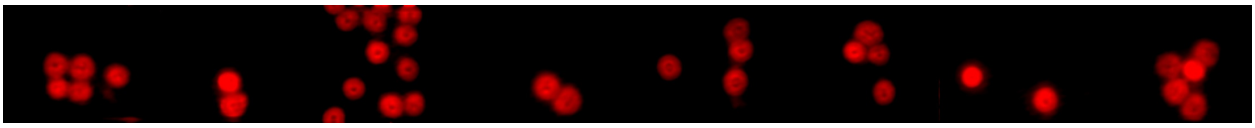


Figure 4.2.5 Large field of view obtained with the help of Scanning Pickup lens in X-axis

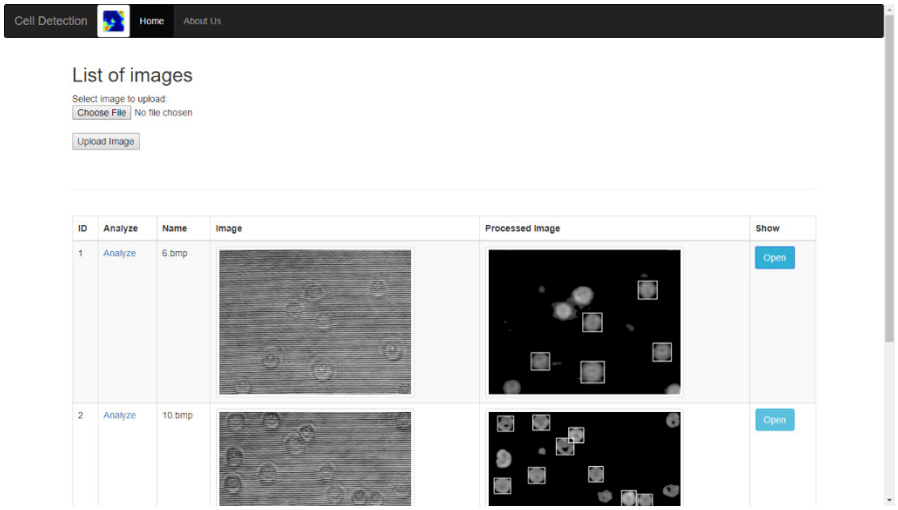


Figure 4.2.6(a) Web-based interface for parameter extraction

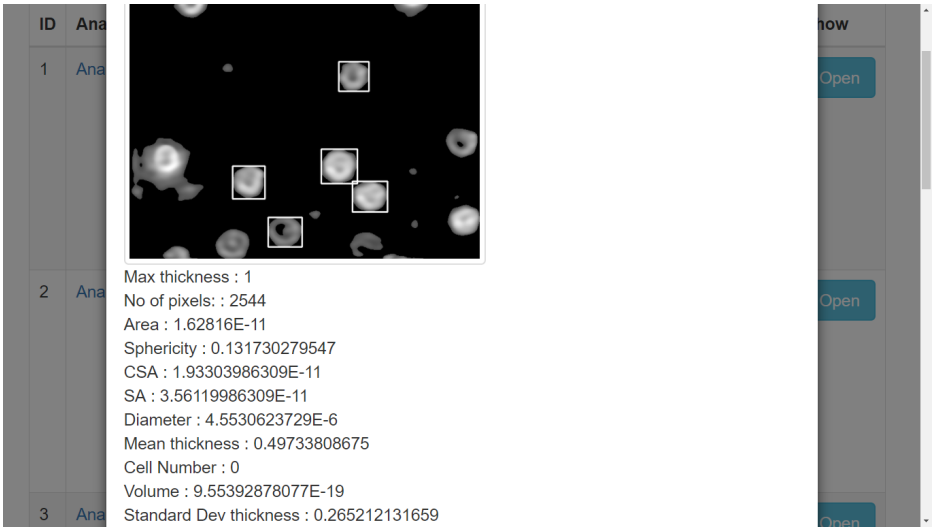


Figure 4.2.6(b) Automated cell parameter extraction

In future, another version of LSDHM can be made with no focusing mechanism and control electronics. The lens is fixed permanently at a specific distance from the sample plane. This makes device simple and with least components. Focusing at the sample plane is done numerically.

4.3 Resolution Improvement and Phase Tomography Using Oblique Illumination

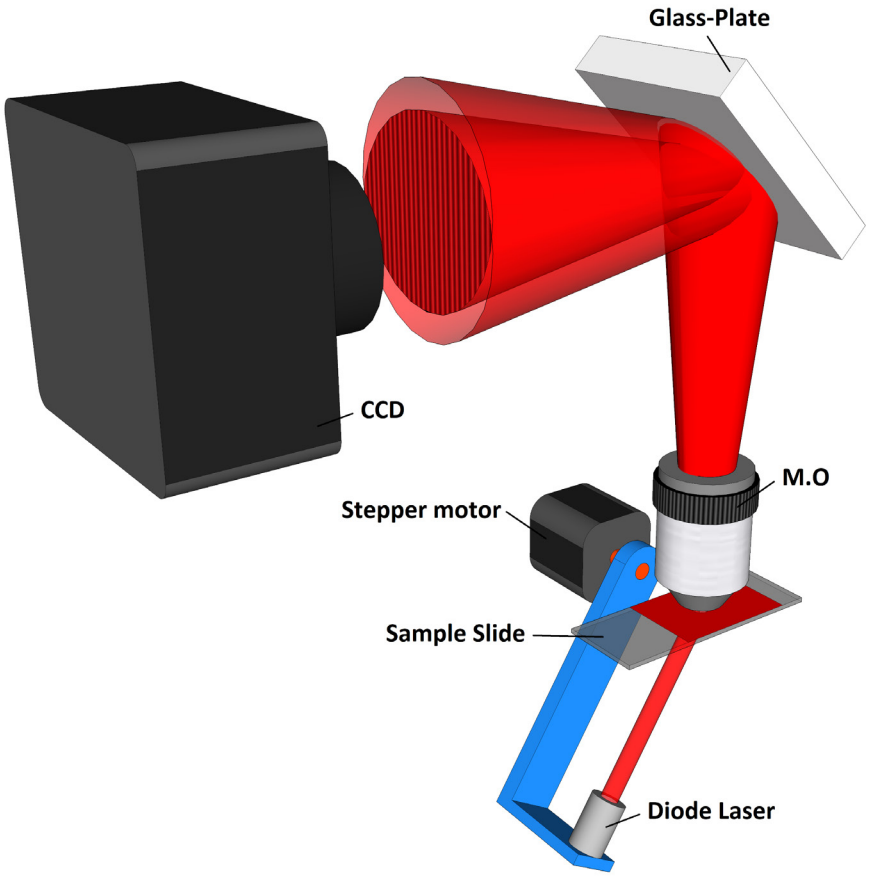


Figure 4.3.1 Schematic of Phase tomography technique using oblique illumination

Phase information attained from DHIM gives only information of average phase value of the sample and lacks information of structures inside the cell. Structures inside of the cell can only be imaged if one can differentiate between regions of different refractive indices in it. The modified lateral shearing DHM technique in which the object is illuminated from different angles and extract phase at those respective angles. Then it is possible to extract thickness profile at different depths of cell at different angles using a back projection tomographic algorithm [53]. Earlier, methods include translation and rotation of the source beam or rotation of sample or tilting of galvanometer mounted mirror to change illumination angle to the sample. But in case of sample rotation, it's difficult to fix the axis of rotation and is typically restricted to solid non-biological objects. So, we have adopted a method in which a laser diode is attached to stepper motor using an arm. The arm sweeps from -36^0 to $+36^0$ making an arc. The sample is kept at the centre of the arc and imaged using lateral shearing DHM configuration. Application of stepper motor gives us ability to illuminate the sample at desired angle without disturbing the sample [54, 55]. The laser diode sweeps the arc with the step of 0.9^0 and hologram were recorded at each step. Set of images from

different angles is used to reconstruct three-dimensional data set using a simple slice-by-slice implementation of the filtered back-projection algorithm.

CHAPTER 5

Digital Holographic Microscope Based on Division of Wavefront

In Wavefront Division digital holographic microscope, A module was designed in which two microscopic objective lenses (Aspheric, FL = 4mm, and N.A. = 0.6) were kept side by side on a platform with 4-5mm gap between them. The sample is kept under one of the lens. The expanding laser beam is directed toward the two-lens system. The two lenses split the beam into two, one portion of beam acts as reference and another portion as object beam. The object beam and the reference beam were made to interfere at the imaging sensor. The advantage of this setup is it has least optical components, lesser complexity, very less flux loss, easy alignment and one of the existing lenses splitting the beam itself act as an imaging lens. Figure [5.1] represents the schematic of the developed setup and figure [5.2] shows the actual setup developed in the lab. To test the imaging capability of the setup, holograms of blood smear on the microscope slide are recorded and the reconstruction is done by ASP approach to digital holography. Also, red blood cells are discriminated using the automated identification algorithm figure [5.3] shows the reconstructed red blood cells.

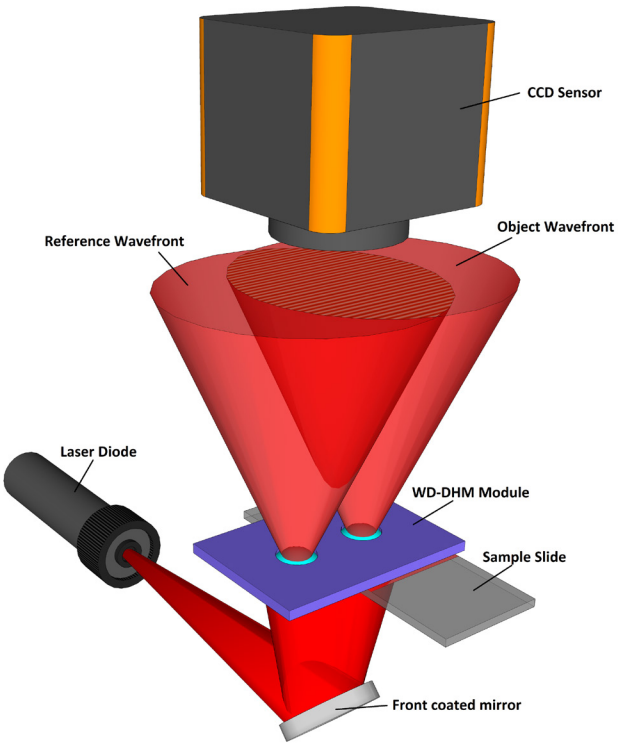


Figure 5.1 Schematic of WD-DHM

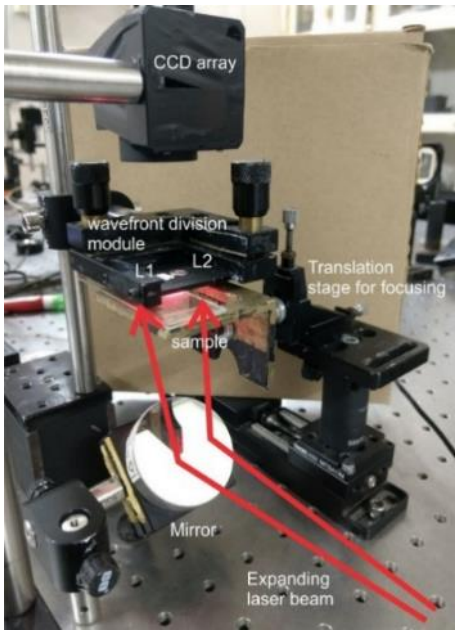


Figure 5.2 Lab Setup of WD-DHM

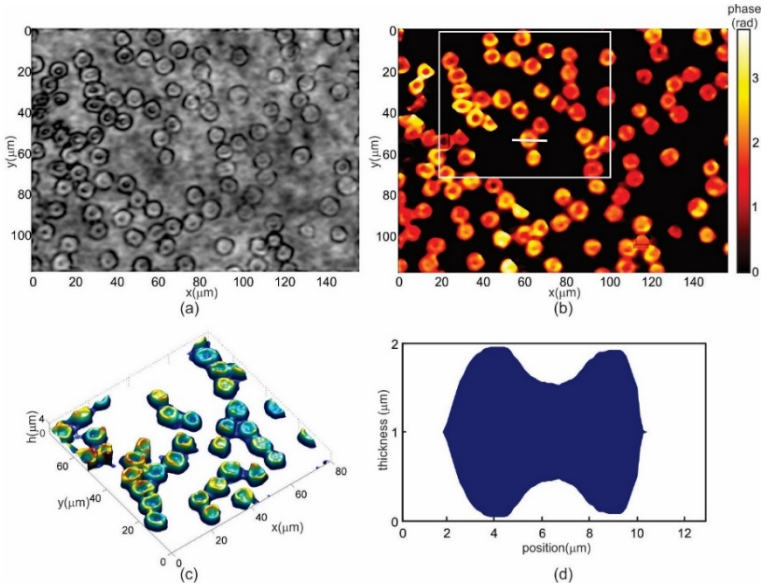


Fig. 5.3(a) Intensity pattern of the human red blood cells obtained from reconstructed holograms.

(b) Quantitative phase image corresponding to the intensity profile shown in Fig. (a).

(c) Thickness profile of blood cells inside the region of interest shown in Fig. (b).

(d) Cross-section thickness profile along the line in Fig. (b).

CHAPTER 6

Combining Low Cost Optical Tweezers with Digital Holographic Microscope for Multiple Parameter Extraction

Optical tweezer [56, 57] is a standard tool to trap and manipulate microscopic objects in three dimensions. Using optical tweezers, one can trap micron size biological [58] as well as technical objects [59]. This technique uses a laser beam that is focused to a near-diffraction limited spot, usually through a high numerical aperture (NA) microscope objective. Micron-sized objects (having refractive index higher than the medium) are trapped at the maximum intensity of the focused laser beam. Single cell immobilization, isolation and sorting are of great interest to biomedical researchers and clinicians. Optical tweezers can be used to study and analyse infected cells [60], tumour cells [61], bacteria [62] and stem cells [63, 64]. Cell sorting can be achieved via various techniques viz. flow cytometry [65], dielectrophoretic trapping [66-68], laminar flow based separations [69, 70] and optical trapping using lasers [69, 71-74]. Most of these mentioned techniques can be difficult to implement practically and sometimes require large volume of sample and/or reagent for analysis. This makes the system complex and expensive and requires skills to record, process and analyse the acquired data.

Optical pickup units found in digital versatile disk (DVD) burners have sufficient laser power and focusing optics to produce a steady Gaussian beam capable of optical trapping [75] and immobilizing biological and technical micro-objects within some size limit. These readily available modules can trap, steer and focus the laser beam simultaneously using the tracking coil contacts and an integrated aspheric singlet objective lens. When combined with DH microscopes [46], this becomes a very potent tool for observing the morphological changes occurring to living cells in their natural environment. We have designed an integrated, stable optical trapping cum 3D imaging device using a DVD optical pickup unit. The quantitative phase imaging of the trapped samples was done using lateral shearing self-referencing digital

holographic microscope. We have achieved stable trapping and 3D imaging of red blood cells and polystyrene beads. The developed technique is inexpensive and uses off-the shelf optical components in comparison with the traditional trapping and 3D imaging apparatus.

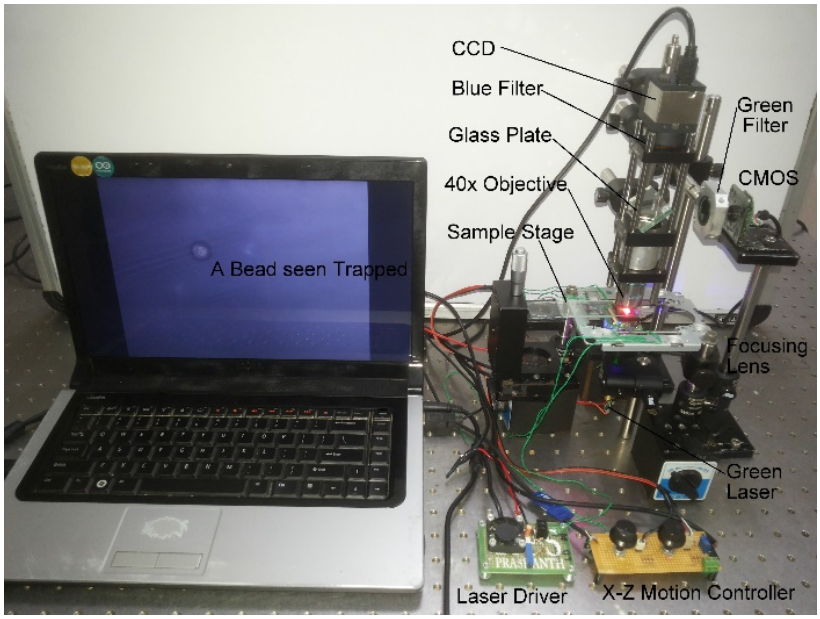


Figure 6.1 Lab Setup of optical trapping

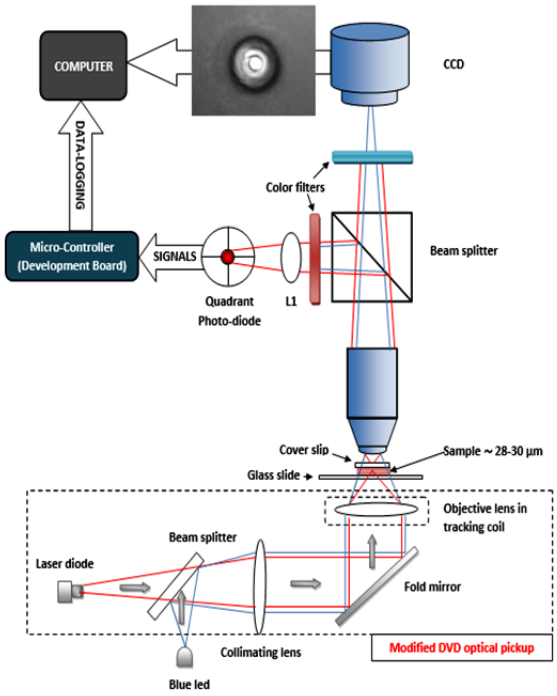


Figure 6.2 Schematic of optical trapping setup

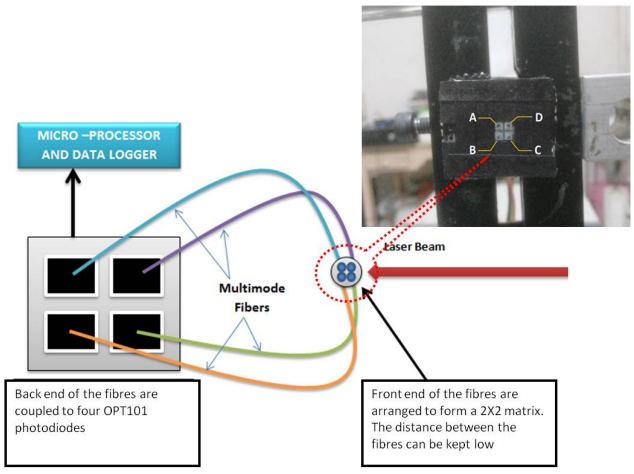


Figure 6.3 Corner frequency measurement using custom developed QPD

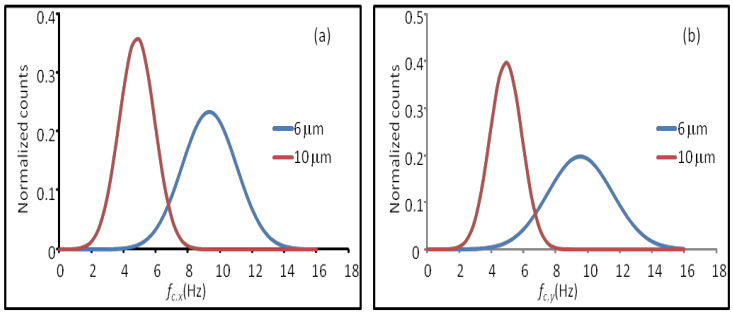


Figure 6.4 (A) Corner Frequency measurement fc,x (Hz) (B) Corner Frequency measurement fc,y (Hz)

Particle size (µm)	Simulation [OTGO] (µN/m)		Experiment (µN/m)	
	k_x	k_y	k_x	k_y
6	4.739	4.725	4.965	5.078
10	2.843	2.834	2.856	2.908

Table 6.1 Corner frequency measurement of 6 and 10-micron polybeads

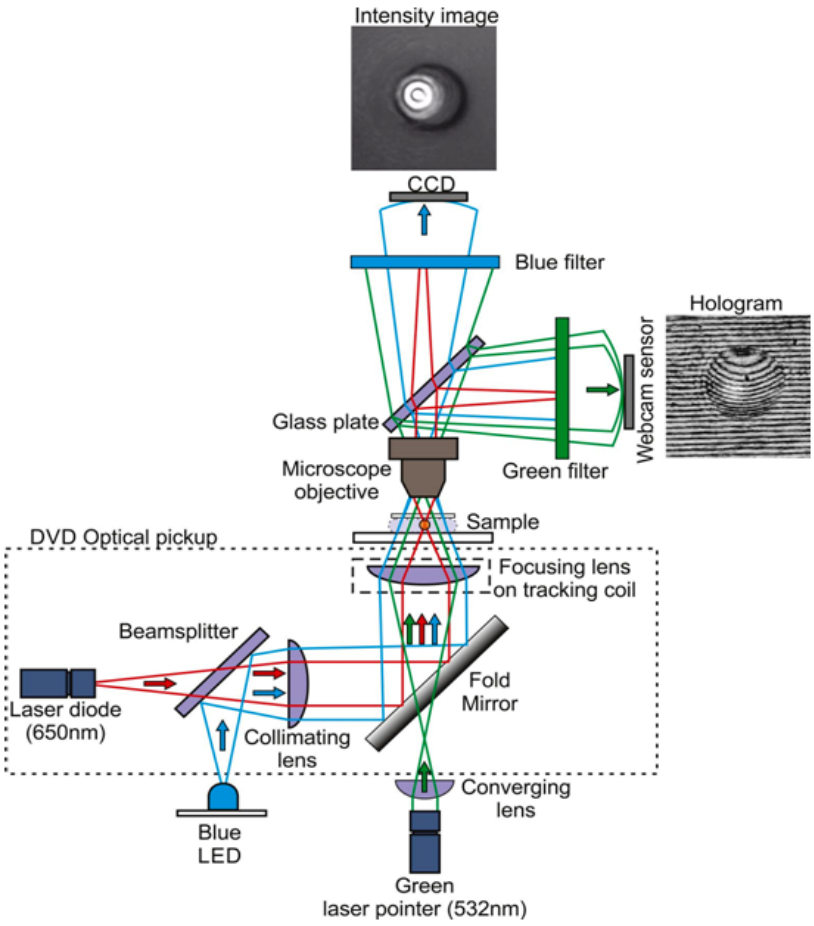


Figure 6.5 Optical trapping setup combined with LSDHM

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