Chapter 4

Two-beam Digital Holographic Interference Microscope for quantifying human erythrocytes

4.1 Human erythrocytes

One of the areas in which the off-axis Mach-Zehnder based two-beam digital holographic microscope would be used is, to measure the parameters related to spatial thickness distribution. These include volume, thickness distribution, shape, surface area, sphericity and ratios of these parameters [20, 22, 25, 26, 27, 31, 34, 36, 45, 78]. Also from a time sequence of holograms (interferograms), optical techniques based on interference can quantify the amplitude and frequency of cell thickness oscillations as well as cell volume fluctuations [27, 42, 72, 73, 74, 75, 77, 82, 84, 108, 109, 110]. This makes quantitative phase imaging an ideal tool for imaging and quantification of blood cells.

Blood cells are unique in the sense that they are few of the cells in human body that can migrate [111, 112, 113]. Blood consists of erythrocytes (red blood cells - RBC), leukocytes (white blood cells - WBC), thrombocytes (platelets) and plasma [113]. Red blood cells are most abundant in blood and it carries oxygen to different parts of the body (while it circulates) and is responsible for expunging carbon dioxide from the body. White blood cells and platelets are less abundant [112, 113]. Diseases affecting the human body, lead to changes in these cells [112]. Hematology analyzers quantify different blood cells in terms of its relative numbers in blood, volume and

distribution [113]. They are very useful in characterization of blood cells (based on the extracted parameters), which may lead to detection or identification of diseases or abnormalities [114]. These devices are based on either flow cytometry (diffraction of light from cells flowing through a narrow tube) or measurement of electrical resistance [115]. As mentioned in Chapter 1 and demonstrated in Chapter 3, MZ-DHIM is an ideal tool for quantifying cells and extracting parameters based on cell thickness. In this chapter, two-beam MZ-DHIM is used for quantitative imaging of human red blood cells and for extraction of the cell parameters.

Human red blood cells do not have a nucleus and a normal RBC has a diameter of 6 μm to 8 μm. They have a discoid shape (bi-concave) and a flexible membrane (Fig.4.1), which allows these cells to pass through narrow capillaries by folding, stretching and bending [113]. It has a cytoskeleton that reinforces the plasma membrane. Hemoglobin is the most important component of red blood cells, which consist of four protein chains (globins). A non-protein component called the heme group is encompassed by each of these chains. Hemoglobin constitutes about 33 percentage of the cytoplasm. Any increase of decrease of hemoglobin, will change the density and hence the volume of the cell. A normal RBC has a volume of around 80 - 90fL which might change in the case of some diseases [27, 72, 73, 74, 82, 108, 109, 112, 113, 114]. This may also be seen in the area and shape of the cell [36]. Cytoplasm in red blood cells are covered by a plasma membrane, which can be considered as stretched above it [116]. When exposed to a concentration or temperature gradient (like the case of thin blood smears on microscope slides), this membrane is set into oscillation. The characteristics of the oscillation (amplitude and frequency) depend sensitively on the elasticity of the layer itself. A measurement of these oscillation parameters might also provide important information about the state of health of the cell [27, 72, 73, 74, 108, 109]. This chapter details the efforts made towards the application of MZ-DHIM for quantitative phase imaging of human erythrocytes.

4.2 Three dimensional imaging of red blood cells

Microscope described in Section 3.2 of Chapter 3 is used for imaging of red blood cells also. In the case of red blood cells, magnification was achieved by the use of

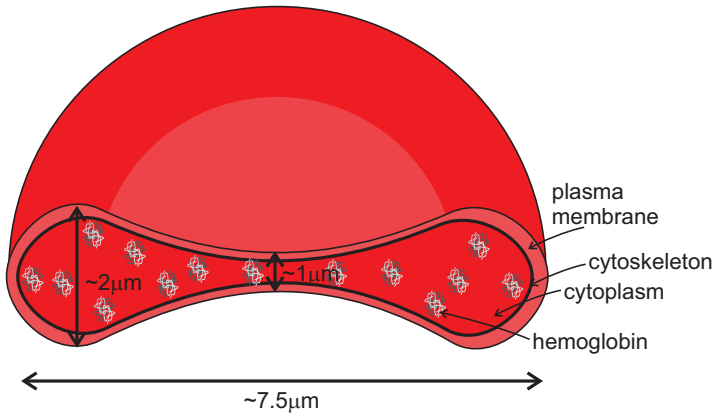

Figure 4.1: Structure of human red blood cell

a 40X, NA=0.65 microscope objective lens and a He-Ne laser working at 632.8nm (power<1mW, random-linearly polarized) was used to illuminate the object. Use of this microscope objective lens provides higher magnification and better resolution. Thin blood smears on microscope slides are used as sample. Hologram of the cells and the surrounding medium (blood plasma) are recorded and is shown in Fig.4.2. These are the object and the background holograms respectively. Fringe modulation can be seen in the regions of cells. Object and background holograms are reconstructed separately. Fig.4.3 shows the reconstructed intensity distribution at the best focus plane. The computed phase difference (Fig.4.4a), brings out the phase information about the object (red blood cells). This wrapped phase distribution is unwrapped and converted to continuous phase distribution, which is shown in Fig.4.4b.

Continuous phase distribution shown in Fig.4.4b is used in Eq. (2.17) to compute the thickness profile (optical thickness profile) of the cells shown in Fig.4.5a. By using an average constant refractive index of the red blood cells and the medium surrounding it (blood plasma), this optical thickness can be converted into real thickness of the cells [25, 27, 36, 117]. Fig.4.5b shows the optical thickness distribution of the cell inside the area of interest (red circle in Fig.4.5a). Cross sectional optical thickness profile of the cell along the line (solid white) in Fig.4.4b is shown in Fig.4.5c, which depicts the donut profile of red blood cells.

From the optical thickness profile many cell parameters can be extracted [118].

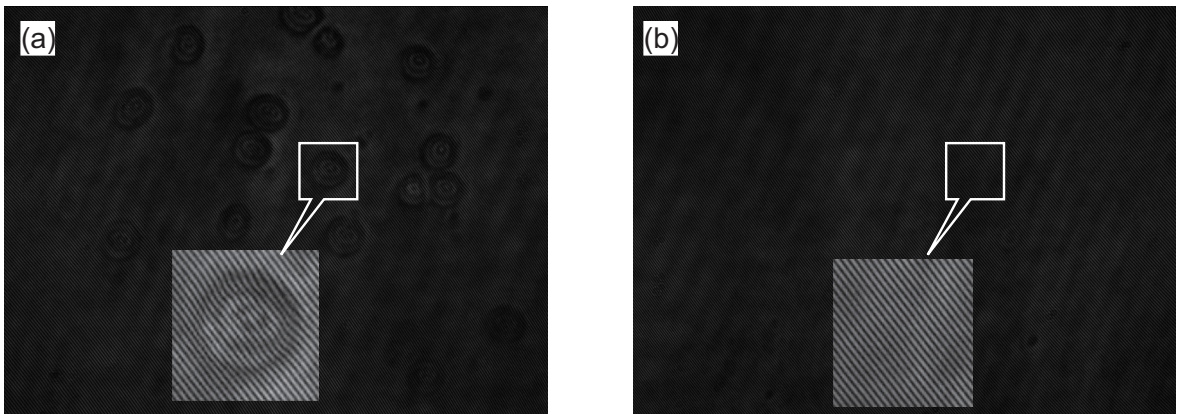

Figure 4.2: Recoded holograms in the case of red blood cells. (a) Object hologram (red blood cells). (b) Background holograms (blood plasma).

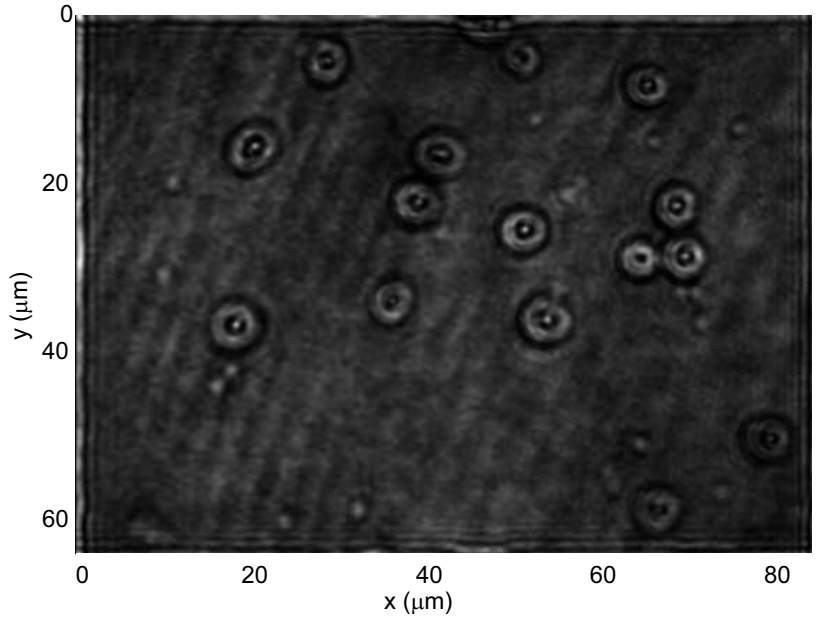

Figure 4.3: Reconstructed intensity profile of red blood cells

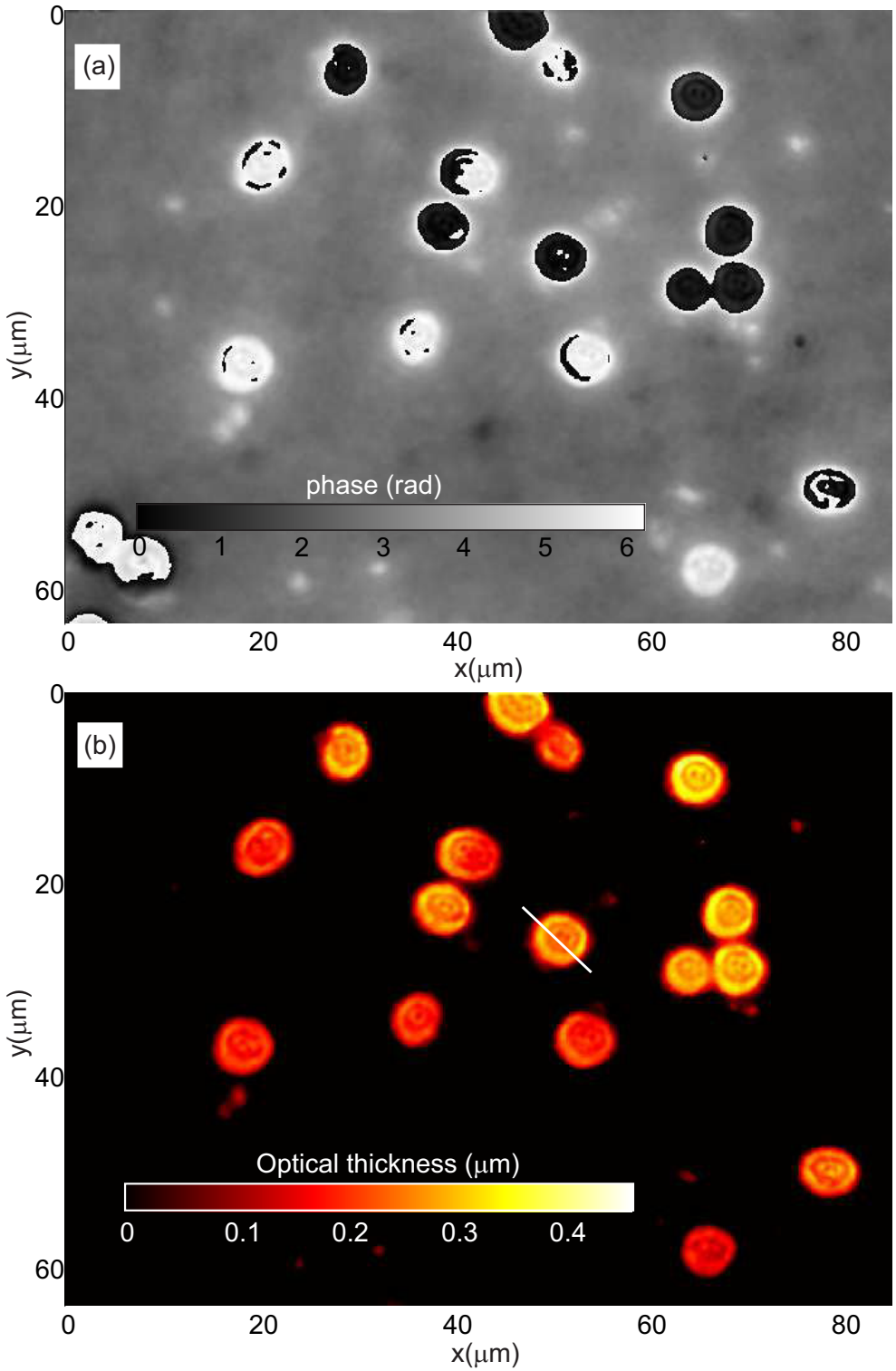

Figure 4.4: Reconstructed profile of red blood cells. (a) Wrapped phase distribution. (b) Optical thickness distribution obtained after phase unwrapping.

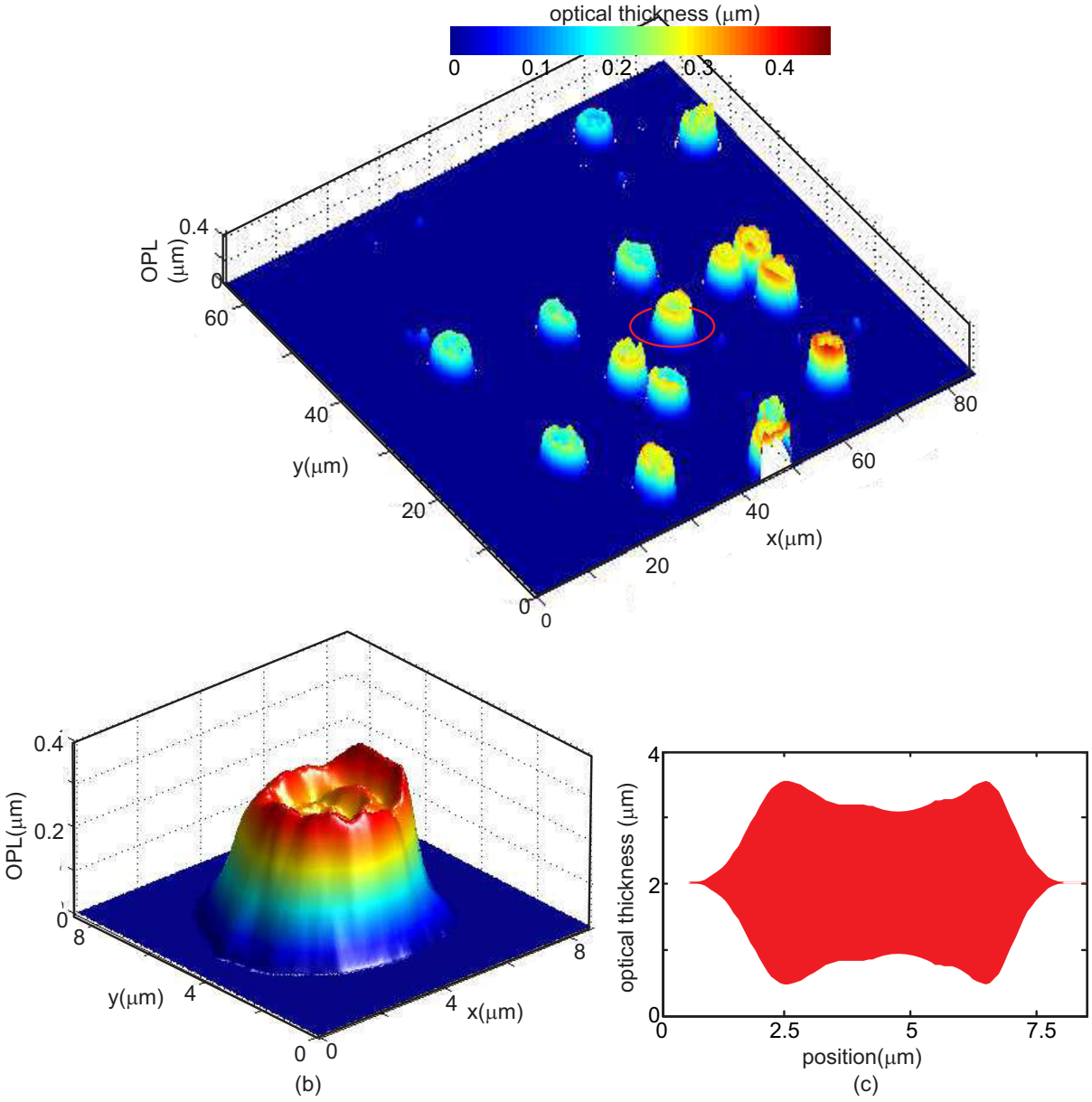

Figure 4.5: Thickness profile of Red blood cells. (a) Computed optical thickness distribution of red blood cells using the reconstructed phase profile in Fig.4.4b in Eqn.(2.5.3). (b) Optical thickness profile of the cell inside red circle in Fig.4.5b. (c) Cross sectional optical thickness profile of the same cell.

The most important of these parameters are (i) thickness (mean, maximum and minimum) and the ratio of thickness distributions in different regions of the cell (for example the outer ring and the inner depression) [44], (ii) Dry mass, which represents the mass of the cell without considering the water content, (iii) Total surface area, which is the sum of the projected area and the surface area of the cell (projected area also provides the diameter of the cell), (iv) Cell volume, which is the sum of the optical thickness multiplied by the projected area [34], (v) Surface area to volume ratio is a very important parameter in the case of most of the cells, especially in the case of red blood cells higher surface area to volume ratio represents higher permeation of oxygen into the cell [113], (vi) Sphericity index and the projected area to volume ratio provides information on flatness of the cell, (vii) Circularity of the cell provides deviation of the cell projected area from circular shape, (viii) Coefficient of variation (CV) of the cell represents the spread of the thickness distribution around the mean value and can also be a measure of the cell flatness [119], (ix) Cell roughness represents the deviation from the mean value (after fitting) of the thickness and it represents the smoothness of the cell [4.33], (x) Shape of the cell can be quantified by comparing it to a predefined shape [36, 119], (xi) Statistical parameters of the cell based on its thickness distribution (skewness and kurtosis) [119].

4.3 Improved temporal phase stability

In the quantification of red blood cells, a compact He-Ne laser source (Thorlabs HNLS800R) is used. This laser source can be placed in the same platform as the microscope, reducing the uncorrelated oscillations. Also, the structure of the microscope was strengthened by connecting different sections of the microscope by posts. Fig.4.6 shows the histogram of the temporal phase stability of the MZ-DHIM used for 3D imaging of red blood cells. For measurement of stability, 1200 holograms at the rate of 20Hz are recorded. The stability of the setup is obtained by the procedure described in Section 3.5 of Chapter 3. Histogram of the fluctuations for the microscope used in 3D imaging of red blood cells is shown in Fig.4.6. Mean value of this provides the temporal phase stability of the system which is about 0.0187 rad. This value used in Eq. (2.17), provides an optical path length stability of 1.88nm.

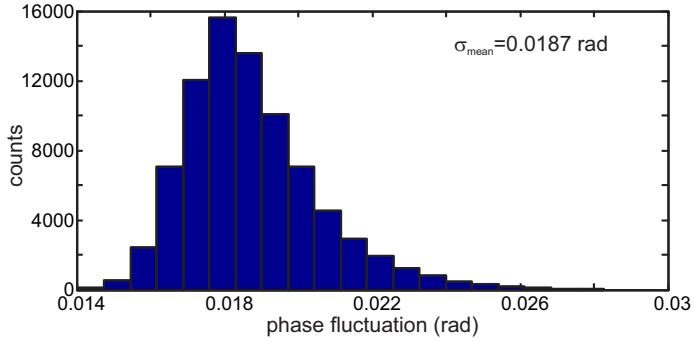

Figure 4.6: Improved temporal phase stability. Histogram of the phase fluctuation obtained for the microscope used for 3D imaging of red blood cells.

4.4 Imaging of red blood cell dynamics

One of the biggest advantages of digital holographic microscopes is that, it can be used to compare the phase of the object wavefront at any two instance of time. With digital holographic microscopy it becomes easy to find the time variations in thickness (morphological changes) occurring to the cell between two time instances. Once the time variations in thickness are quantified, these could be used to extract parameters (like amplitude and frequency of thickness fluctuation) of the cell that depend on the time variation of thickness [27]. To measure the cell thickness fluctuations, holograms of thin blood smears on microscope slides are recorded at the sampling rate of 20Hz for a period of 30s. Microscope slide containing the thin smear is allowed to settle for two minutes before the holograms are recorded. Phase fluctuation at each spatial point can be written as

$$\Delta\phi_{fluct}(x,y) = \sqrt{\frac{1}{N}\sum_{t=1}^{N}\left[\Delta\phi(x,y,t) - \langle\Delta\phi(x,y,t)\rangle_{time}\right]^2} = \frac{2\pi}{\lambda}\Delta n \times L_{fluct}(x,y) \tag{4.4.1}$$

In the above equation N is the number of holograms (number of time points), $\Delta\phi(x,y,t)$ is the phase difference at a spatial point at an instance of time, $\langle\Delta\phi(x,y,t)\rangle_{time}$ is the time average of the phase variation and $\Delta n \times L_{fluct}(x,y)$ is the optical thickness fluctuation at each spatial point. Fig.4.7 shows the phase fluctuation profile computed from the reconstructed phase difference distributions using Eqn.(4.4.1). Color bar on

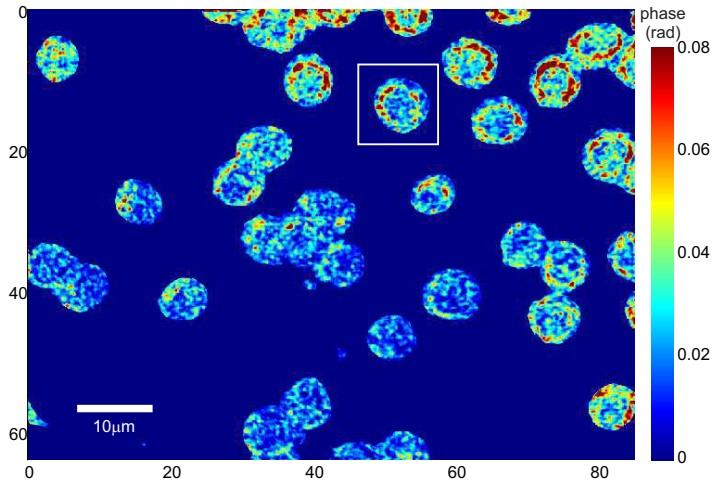

Figure 4.7: Phase fluctuation profile of red blood cells quantified using MZ-DHIM. This is computed using 600 holograms recorded at the rate of 20Hz.

the right side of Fig.4.7 indicates that the fluctuations are higher near the periphery of the cells which might be due to lateral movement of the cells [73]. The minimum measurable phase fluctuation depends only upon the temporal phase stability. This is decoupled from the lateral resolution, which depends upon the numerical aperture of the magnifying lens.

Contour plots of time variation of phase for the cell inside the white rectangle in the above figure is shown in Fig.4.8. Standard deviation along the time axis at each spatial point provides the phase fluctuation at that point by the use of Eqn.(4.4.1). Fig.4.9a shows the phase map of the red blood cell. Optical thickness of the cell is proportional to this phase distribution through Eqn.(2.5.3). Fig.4.9b shows the phase fluctuation map of the same cell computed for a period of 30s (600 holograms). It can be clearly seen that the fluctuation are high near the periphery of the cell. This is also evident from the 3D rendering of the optical thickness fluctuation profile shown in Fig.4.9c. Time variation of the optical thickness (at the points indicated in Fig.4.9a) is shown in Fig.4.10 along with their standard deviations (optical thickness fluctuation). It can be seen that the optical thickness fluctuation depends upon the position of the spatial point on the cell. Fig.4.9b and Fig.4.9c indicates the existence of sub-domains around the cell structure [73]. The variation in the optical fluctuation

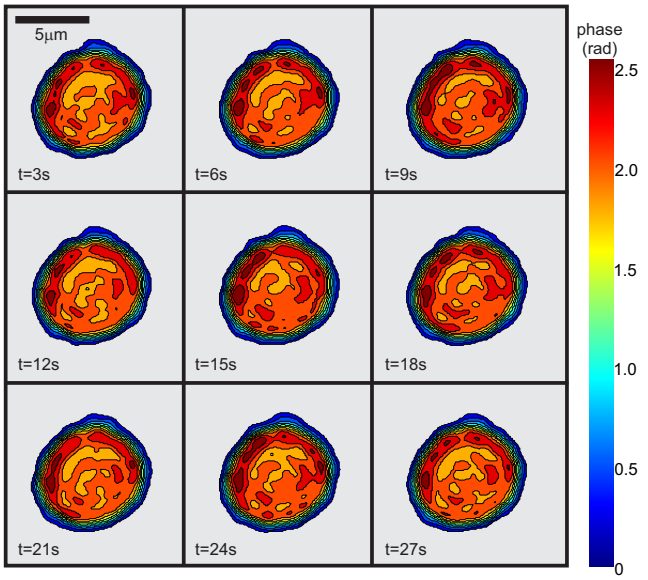

Figure 4.8: Time varying phase profile of red blood cell (for the cell inside the white rectangle in Fig.4.7

profile can be observed by finding the standard deviation of the time varying thickness profiles for shorter durations. Fig.4.11 shows the time variation in the domains across the cell. This is obtained by finding the fluctuation profile for every 3s (60 time points or 60 holograms). The time variation of the structures inside the cell is quite evident from this figure and the size and coherence of the domain can be determined.

The dominant frequencies of the sub-domains shown in Fig.4.9 can be determined by Fourier transforming the time evolution of the optical thickness variation. Fig.4.12 shows the frequency spectrum of the path length variations shown in Fig.4.10. The peak frequency at each spatial point is determined from the frequency spectrum. Fig.4.13 shows the spatial variation of the peak frequency across the entire cell.

The relation between the amplitude and frequency of thickness fluctuations with time is shown in Fig.4.14. It indicates a strong relationship between them. These parameters can be used to study the bio-physical properties of the cells. Reconstructed optical thickness distribution can be used to extract different cell parameters, which depend upon its morphology. Table 4.1 tabulates the various static and dynamic

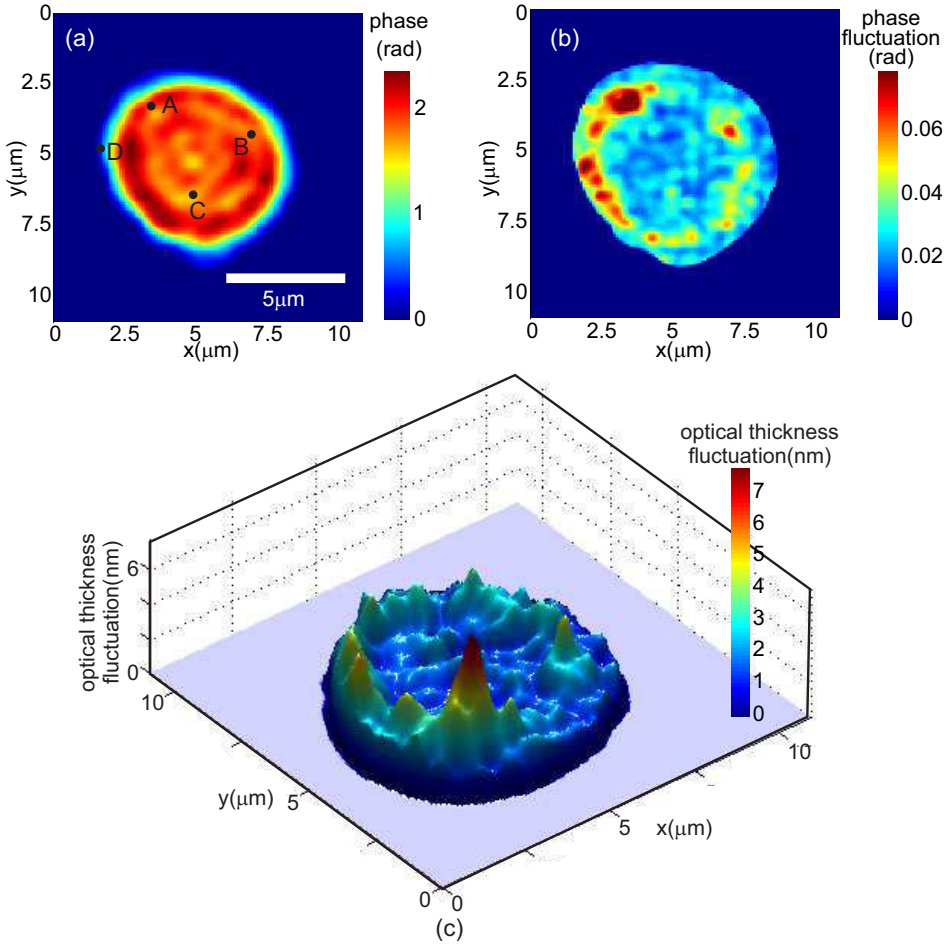

Figure 4.9: Phase, phase fluctuation and optical thickness fluctuation amplitude of red blood cell inside the area of interest shown in Fig.4.7. (a) Phase profile of the cell. (b) Phase fluctuation profile of the cell. (c) Optical thickness fluctuation profile of the cell.

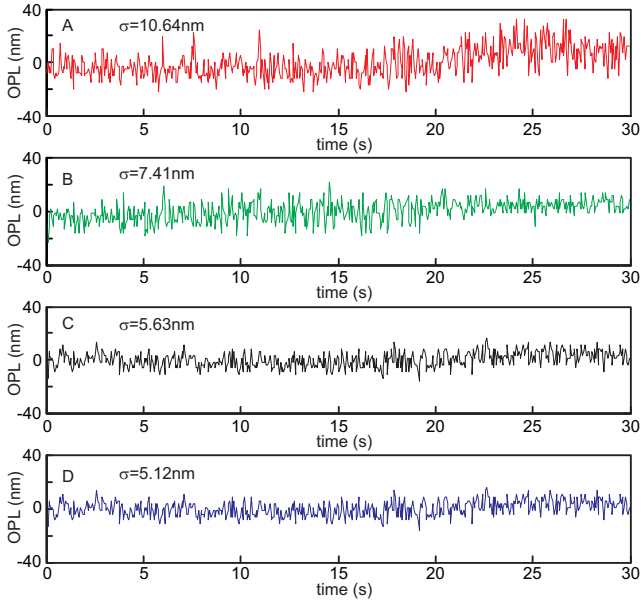

Figure 4.10: Time variation of optical thickness (OPL). Temporal evolution of optical thickness at the points indicated in Fig.4.9a along with the standard deviation of each plot (which indicates the optical thickness fluctuation at that point).

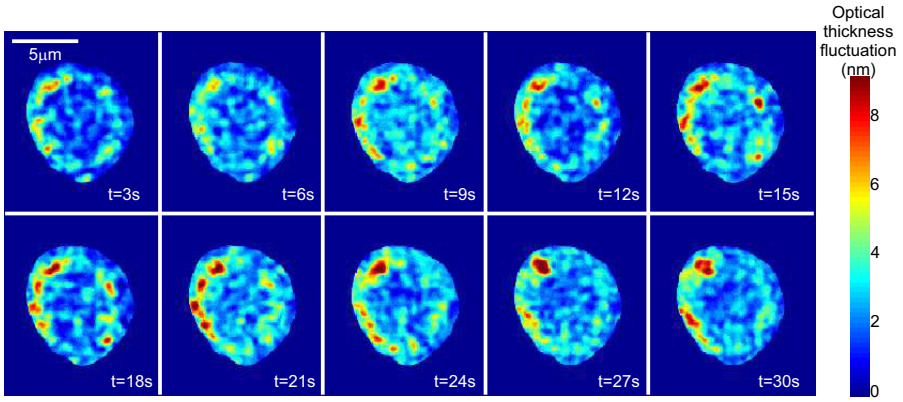

Figure 4.11: variation in optical thickness fluctuation amplitude across the whole cell as a function of time. Dynamic sub-domain across the cell can be seen in the image. These can be quantified and their coherence (temporal and spatial) can be determined.

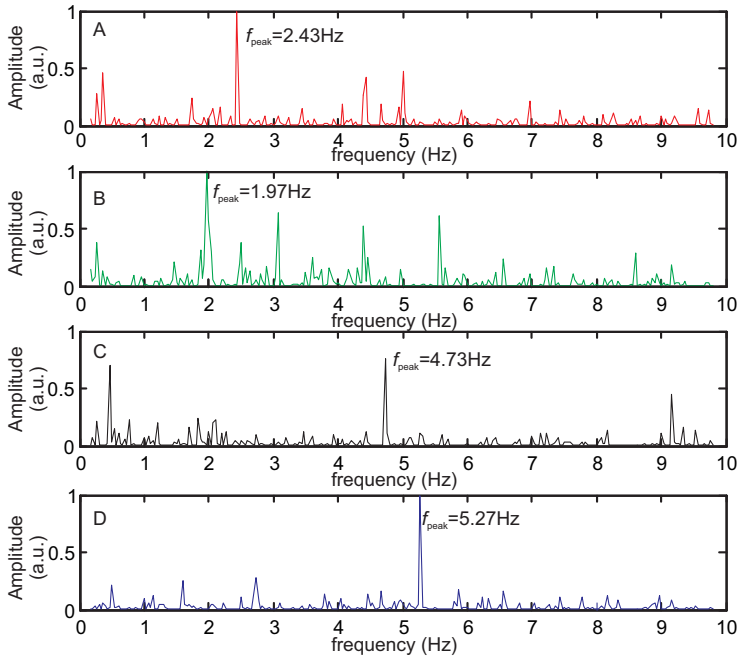

Figure 4.12: Frequency spectrum of time variation of optical thickness (OPL). Frequency spectrum of the optical path length change for the points shown in Fig.4.9a

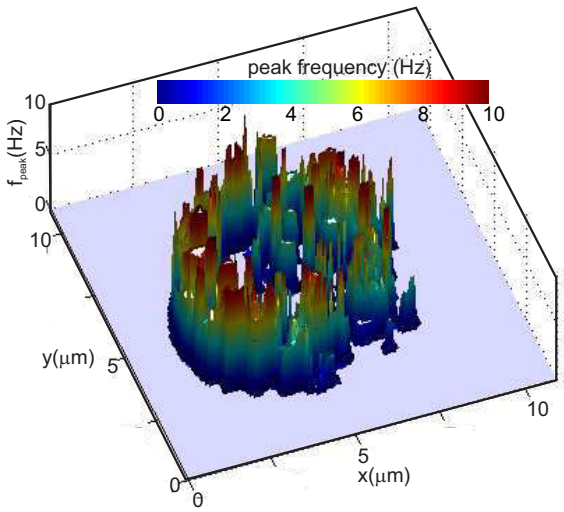

Figure 4.13: Distribution of thickness fluctuation frequency across the cell.

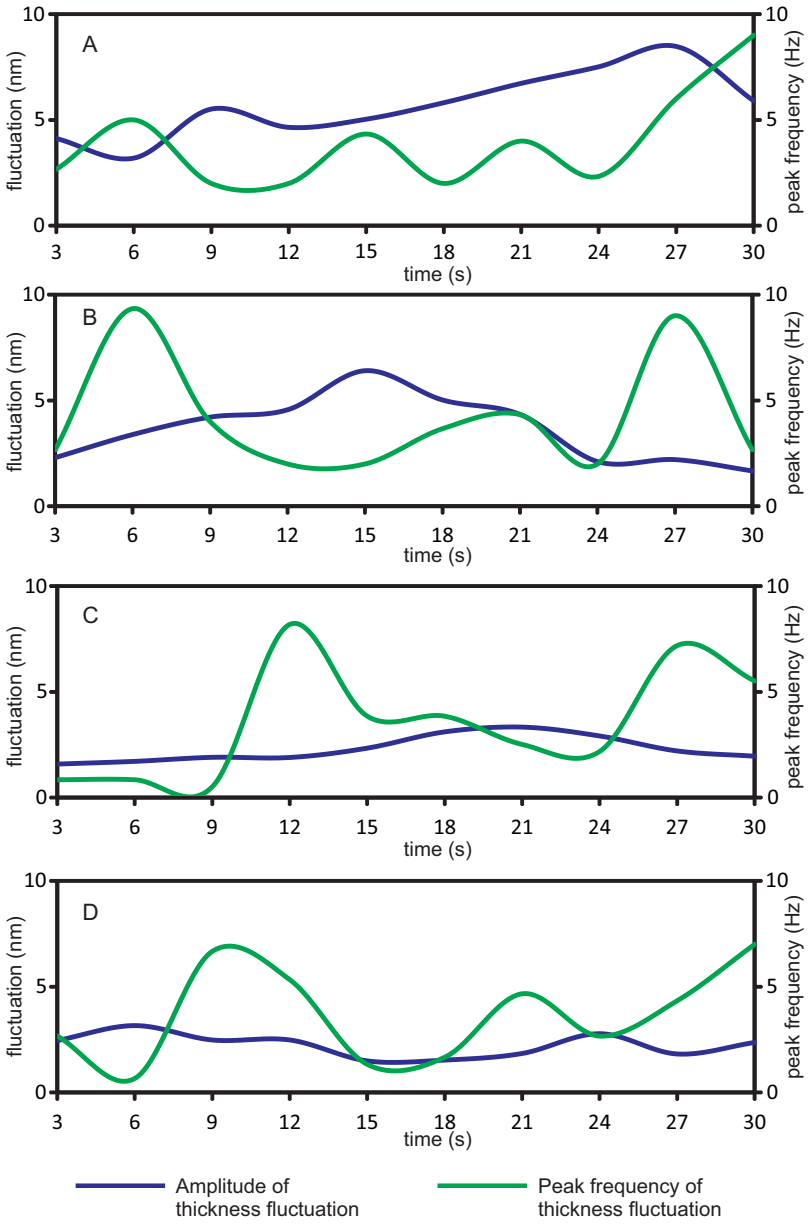

Figure 4.14: Time evolution of amplitude and frequency of optical thickness fluctuation at different points on the cell shown in Fig.4.9a

Table 4.1: Static and dynamic parameters of healthy red blood cells.

Sr. No.	Parameter	Measured value (mean±std)
1	Diameter	7.54±0.42 μm
2	Mean value of optical thickness	0.312±0.088 μm
3	Coefficient of variation of optical thickness	0.1819±0.042
4	Projected area	44.84±5.21 μm^2
5	Optical volume	7.12±1.21 fL
6	Optical total surface area	91.22±11.31μm^2
7	Surface area/optical volume ratio $\times 10^6$	12.54±0.61 m^{-1}
8	Sphericity	0.261±0.021
9	Amplitude of thickness fluctuation	2.61±0.86nm
10	Mean peak frequency of thickness fluctuation	3.73±1.41Hz

parameters of red blood cells obtained using MZ-DHIM. These parameters were determined using 50 cells.

These parameters are similar to the ones obtained with hematology analyzers, but using a much simpler, compact and economical setup. These parameters will be helpful in identifying diseases affecting red blood cells like sickle cell anemia, malaria, and thalassemia [36, 119]. In presence of external force or stresses or in various pathophysiological conditions, deformability of RBC (ability to change shape in response to some internal or external force) gets altered. Cell geometry, temperature, calcium concentration, aging of cells, hereditary disorder and many other factors play key role in alteration of deformability of RBC. Altered deformability of RBC is an important factor that may change blood viscosity and thus blood circulation. This deformation can cause change in cell parameters like curvature of cells, area expansion, volume change or changes in its dynamics. So understanding of deformability of RBC in various conditions is crucial in gathering information about RBC related diseases. Abnormalities in morphology and mechanical properties of RBC such as change in shape and size of red blood cells, change in shape of central pallor of RBC may also be due to some disease or deficiencies in the body [112, 113, 114]. Thus analysis of red blood cells using parameters extracted from their morphology, which is sensitive to change in internal or external conditions of cells may reveal many facts related to its state of health. MZ-DHIM has achieved this goal to a great extent.