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Compact, common path quantitative phase microscopic techniques for imaging cell dynamics

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Abstract. Microscopy using visible electromagnetic radiation can be used to investigate living cells in various environments. But bright field microscopy only provides two-dimensional (2D) intensity distribution at a single object plane. One of the ways to retrieve object height/thickness information is to employ quantitative phase microscopic (QPM) techniques. Interferometric QPM techniques are widely used for this. Digital holographic microscopy (DHM) is one of the state-of-the-art methods for quantitative three-dimensional (3D) imaging. Usually it is implemented in two-beam geometry, which is prone to mechanical vibrations. But to study dynamics of objects like red blood cells, one needs temporal stability much better than the fluctuations of the object, which the two-beam geometry fails to deliver. One way to overcome this hurdle is to use self-referencing techniques, in which a portion of the object beam will act as the reference beam. Here the development of self-referencing QPM techniques is described along with the results.

Keywords. Quantitative phase contrast imaging; digital holography; cell imaging; diffraction; three-dimensional microscopy.

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1. Introduction

Bright field microscopy is an ideal tool for non-invasive monitoring of living cells under various conditions. But many living cells have low absorption for electromagnetic radiation in the visible regime and hence are transparent to visible light. So bright field microscopy yields only low contrast images which has only few useful details about the cell. One of the typical practices used to increase the contrast of semitransparent cells is to

stain and fix them with high absorption coefficient dyes. Unfortunately, this is an invasive process that may terminate/alter the cell's life cycle. However, cell biologists typically need to monitor live cells to study their behaviour and dynamics. One way to overcome this hurdle is to image the phase of the probe wavefront. Phase of the probe radiation is very sensitive to the optical path length mismatch between the cell and the surrounding medium. This property has been used in conventional phase contrast as well as differential interference contrast (DIC) microscopic techniques to create high-contrast intensity images [1–4]. But such techniques provide only qualitative information about the object (cell thickness/refractive index information as a change in intensity). These methods do not provide direct access to the information about the object's spatially varying optical thickness. Also the use of microscopic objective lenses having high magnification and high numerical aperture require mechanical scanning to bring different sections of the object into focus. This increases the imaging time and makes it impossible to study dynamic events. Quantitative phase contrast techniques, which directly provide information about the phase of the object wavefront, can be used to quantitatively image the object under investigation. Typically, interferometric techniques are used for quantitative phase imaging.

2. Digital holographic microscopy

Holograms are interference patterns resulting from the superposition of an object wavefront (wavefront interacting with the object) with a reference wavefront [5]. These are recorded on photosensitive medium such as photographic plates. They contain the information about the object wavefront in the form of a spatially varying intensity pattern (interference fringes). Holograms are reconstructed by illuminating it with the reference wavefront. Diffraction of light from the microstructures of the hologram results in the whole-field (amplitude and phase) reconstruction of the object wavefront [5]. In digital holography, the interference patterns or holograms are recorded on pixilated semiconductor arrays and are reconstructed numerically by simulating the diffraction of the reference beam from the microstructures of the hologram [6,7]. Numerical reconstructions directly provide the complex amplitude of the object wavefront, which in turn provides information about its phase and amplitude.

Digital holographic microscopy (DHM) is a non-invasive, quantitative, single-shot, phase imaging method which provides information about the optical thickness of live cells under dynamic conditions using the principle of digital holography [8–17]. Optical thickness, which is obtained from the phase of the reconstructed object wavefront, is related to cell thickness and its morphology through the refractive index of the cell and the surrounding medium [17].

It should be noted that hologram recording process is a single-shot process and the reconstruction yields the phase of the object wavefront. Thus, when coupled with appropriate reconstruction algorithms, DHM can be an attractive candidate for non-invasive real-time 3D imaging of cells and microorganisms. One of the easiest ways to construct DHM is by using in-line geometry. Even though this geometry is easy to construct and can be implemented using low coherent sources, it suffers from twin-image problem (the real, virtual images and the undiffracted waves are superposed during reconstruction)

[5–7]. So, retrieving phase information from in-line holograms requires additional optics for phase shifting [8] or the recording of multiple holograms. The problem of the twin images can be eliminated by employing off-axis geometry, where the object and reference beams are made to interfere at an angle. In the reconstruction, the real, virtual images and the undiffracted reference beam will be spatially separated [6]. This makes it possible to directly access the object phase information. Figure 1a shows the schematic of an off-axis DHM. Microscopic objective in the reference arm is used to match the curvatures at the recording plane. Figures 1b and 1c show the recorded hologram of a red blood cell using a 100× oil immersion objective lens (NA=1.25) and an 8-bit CCD chip with 4.65 μm pixel pitch [17]. Reconstruction of the recorded digital holograms is achieved by simulating diffraction of the reference beam occurring at the microstructures of the recorded hologram using scalar diffraction integral [15–17]. Since the digitally recorded holograms are discrete in nature (finite pixel and array size), discrete form of the diffraction integral is used. Also here, since short-distance propagations (usually image plane is situated very close to hologram plane) are involved, angular spectrum approach to the diffraction theory was used for reconstructions [18]. For microscopy of phase objects, the angular spectrum propagation (ASP) approach also makes a compact experimental set-up possible. An added advantage of this method is that it can separate out the different diffracted components in the frequency domain, and hence, there will not be any overlap between these components at the reconstruction plane [15–17]. The reconstructed complex amplitude yields the intensity and information about the object (figures 1d and 1e). In figure 1e, phase information obtained from the hologram of the background (recorded without the object, but with the surrounding medium present) is subtracted from the object phase information to remove most of the system aberrations and this provides information about the object thickness through [17]

$$\Delta\phi\,(x, y, t) = \frac{2\pi}{\lambda}\,(n_{\mathrm{o}} - n_{\mathrm{r}})L(x, y, t)\,, \tag{1}$$

where $\Delta\phi$ is the computed phase difference, n_{o} and n_{r} are the constant refractive indices of the object and the surrounding medium respectively and L is the spatially varying thickness profile of the object at time t. Figures 1f and 1g show information about the thickness of the obtained object. Off-axis two-beam DHM directly provides phase information about the object, but it is achieved by combining a separate reference wavefront with the object wavefront at an angle, requiring additional optics (for splitting the beam) and careful adjustment of intensity ratios (for right fringe contrast) as well as vibration isolation (to avoid unwanted path length variation along both beams).

3. Self-referencing DHM

The main disadvantage of DHM employing Mach–Zehnder configuration, is that it is prone to mechanical vibrations due to the two-beam geometry. When two beams travel along different paths, they can acquire phases (due to vibration), which are uncorrelated, leading to low temporal stabilities. This affects measurement of dynamic events like cell membrane fluctuations. To overcome this hurdle, one would like to have a single path set-up (like that of in-line DHM) providing direct access to phase information (like that of

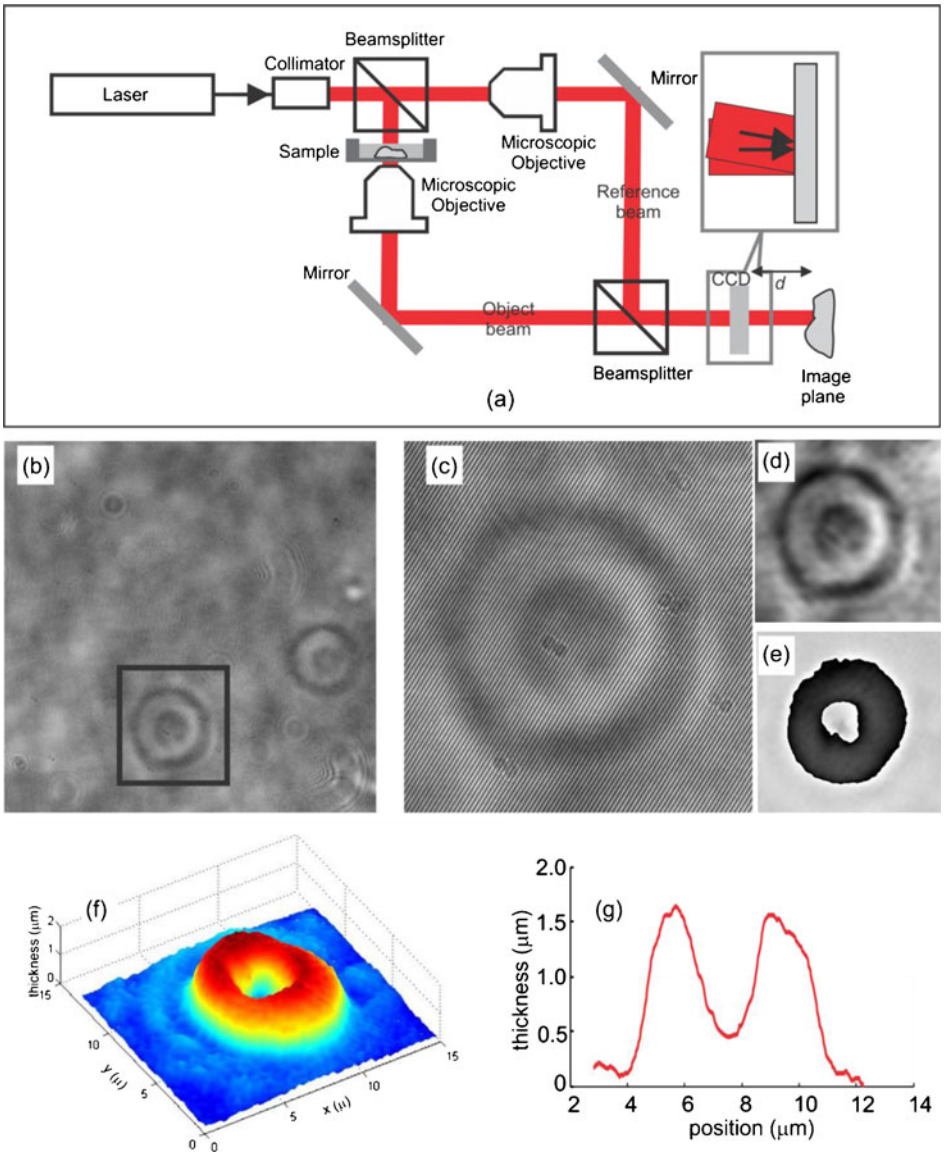

Figure 1. (**a**) Two beam off-axis DHM, (**b**) recorded hologram, (**c**) interference pattern inside the marked zone, (**d**) reconstructed intensity profile, (**e**) reconstructed phase profile after phase subtraction, (**f**) thickness profile obtained from the phase using eq. (1), (**g**) cross-sectional thickness profile of the cell.

off-axis DHM). Unwanted fluctuations arising due to two-beam geometry can be greatly reduced by using common path geometry [19–22]. Another easier way to improve the stability is to employ self-referencing, in which a part of the object beam, that does not exhibit sample structure, acts as the reference beam [23–27]. The advantage of this class

of imaging techniques is that they do not require any special optical element to convert the object beam into a reference beam.

One of the easiest ways to create a self-referencing DHM is to use a glass plate to create two laterally sheared versions of the object wavefront, which are made to superpose at the sensor plane (figure 2a) [26]. Given that the size of the object is smaller than the shear and the beam is large enough, the portion of the beam that contains object information and the unperturbed part can interfere forming a hologram (figure 2b). The recorded interferograms using the lateral shearing method are equivalent to off-axis holograms and are processed accordingly. The phase information from the hologram of the background (recorded without the object, but with the surrounding medium present) is subtracted to get object phase information. The reconstructed phase distribution along with refractive index values of the material of the glass bead and the surrounding medium was used to

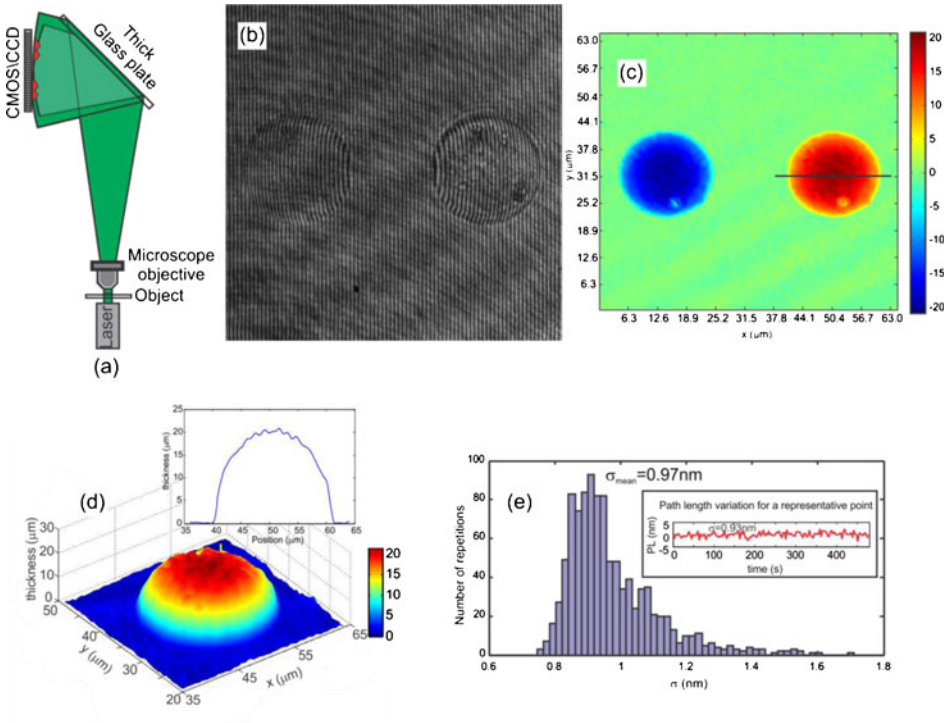

Figure 2. (**a**) Schematic of DHM using lateral shearing geometry employing a glass plate. (**b**)–(**e**) Experimental results for self-referencing DHM using a glass plate. Object used was a glass bead with 20 μm diameter. A 633 nm He–Ne laser was used as the source and an 8-bit CCD with 4.4 μm pixel pitch served as the detector. (**b**) Recorded hologram of the object, (**c**) the obtained thickness distribution of the investigated bead from the phase map, (**d**) 3D rendering of the thickness profile of the right reconstructed image in figure 2b. Inset shows 1D thickness profile along the line in figure 2b, (**e**) standard deviations of path length variations (fluctuations) at 1024 randomly selected pixels within the field of view over 8 min. Inset shows variation of a representative point over time.

reconstruct the thickness profile of the bead using eq. (1) and are shown in figures 2c and 2d. The most important advantage of this technique is its immunity to external vibrations. To determine the temporal stability of the set-up, a series of holograms were recorded, with only the glass slide present (1200 holograms at 2.5 Hz). The stability of the set-up is determined from the mean fluctuation (which is the standard deviation of the thickness variation). From figure 2e it can be seen that temporal stability of the set-up is better than 1 nm over a period of 8 min. The ability of the set-up to image biological specimen was tested using red blood cells. Holograms of thin blood smears were recorded and reconstructed and the obtained results are shown in figure 3. In this case, a 5 mW diode laser at 532 nm acted as the source and a CMOS sensor with 8-bit dynamic range and 5.2 μm pixel pitch was used as the detector.

The two disadvantages of this method using the glass plate are: (i) to change the fringe density, which is important in many cases, one has to replace the glass plate and (ii) there is loss of flux. To overcome these problems, self-referencing technique can be implemented by using wavefront division geometry, by folding a portion of the object beam back onto itself using a tilted mirror as shown in figure 4a [27]. The measured temporal stability for this technique from 300 holograms over a period of 10 min was 0.9 nm. Self-referencing QPM can be used to image fluctuations of cell membranes. From a series of recorded holograms, time-varying thickness profile of the cell can be obtained. This can then be used to determine the membrane fluctuations for each point of cells. So, using self-referencing techniques, membrane fluctuations across the cell can be mapped. Figures 4b–4e show the membrane fluctuations measured for a normal human red blood cell. The set-up employed the self-referencing configuration employing Lloyd's mirror configuration [27]. It used a 45× microscopic objective with NA=0.65. Holograms were recorded for 5 min at the rate of 1 Hz (total of 300 holograms) using an 8-bit CMOS sensor with a pixel pitch of 5.2 μm. A commercial-grade 532 nm diode laser was used as the source. A background hologram (hologram of the region in the slide where there was no cell) was also recorded. Numerically reconstructed phase of each object hologram was subtracted from the obtained phase of the reference hologram to get the object phase information. This was converted to the cell thickness using the average constant refractive indices for the cell and the background medium using eq. (1). The time evolving thickness

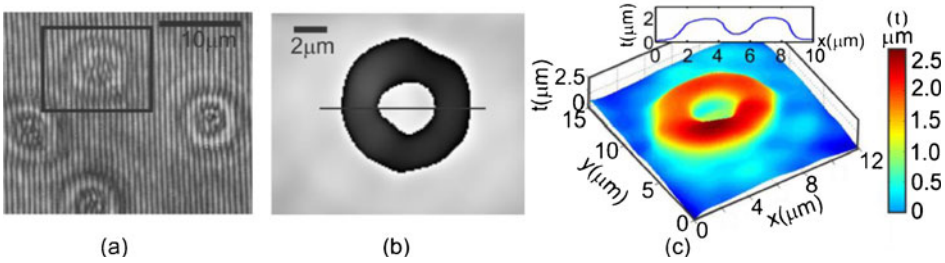

Figure 3. Experimental result of imaging RBCs with lateral shearing DHM. (**a**) Hologram, (**b**) reconstructed phase of highlighted RBC in (**a**), (**c**) 3D rendering of thickness distribution. Inset shows the profile along the line in (**b**).

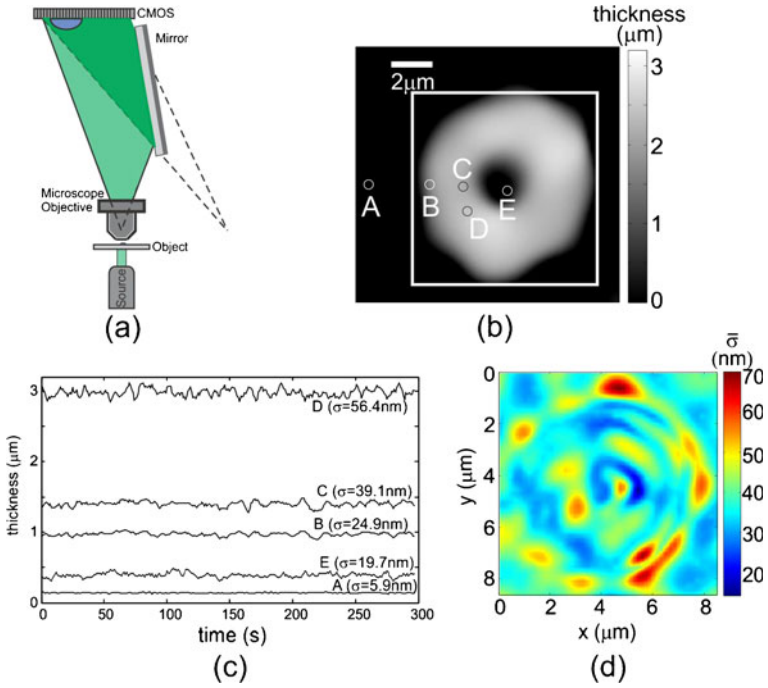

Figure 4. (**a**) Self-referencing DHM using wavefront division geometry, (**b**) phase contrast image of the analysed RBC, (**c**) temporal evolution of cell thickness at points shown in (**b**), (**d**) mean fluctuation map of the cell. Standard deviations of the fluctuations are also shown.

information (figure 4c) was used to determine the membrane fluctuation map of the cell shown in figure 4d.

4. Conclusions

DHM employing self-referencing concept has many advantages over the two-beam technique, like compactness, high stability and cost-effectiveness. It can be used to measure cell dynamics with temporal stability less than 1 nm. The obtained 3D imaging as well as stability and dynamic imaging capability results show that DHMs, especially self-referencing ones, emerge as an ideal label-free tool for investigating static and dynamic phenomena in low absorbing micro-objects including living cells. These techniques can be exploited further for recognition/identification and comparison of cells.

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