Quantitative phase imaging of biological cells using spatially low and temporally high coherent light source

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In this Letter, we demonstrate quantitative phase imaging of biological samples, such as human red blood cells (RBCs) and onion cells using narrow temporal frequency and wide angular frequency spectrum light source. This type of light source was synthesized by the combined effect of spatial, angular, and temporal diversity of speckle reduction technique. The importance of using low spatial and high temporal coherence light source over the broad band and narrow band light source is that it does not require any dispersion compensation mechanism for biological samples. Further, it avoids the formation of speckle or spurious fringes which arises while using narrow band light source. © 2016 Optical Society of America

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Optical phase microscopy is broadly classified into two categories: qualitative phase imaging and quantitative phase imaging (QPI) techniques. The bright-field, dark-field, differential interference contrast (DIC), phase contrast (PC) microscopic techniques [1] give only qualitative information about biological objects or samples, such as shape and relationships to other objects. Moreover, some of the DIC techniques can also provide the quantitative information about the biological specimens [2–4]. In contrast, QPI techniques provide the measurement of different parameters associated with biological objects, such as cell dynamics (i.e., thickness and refractive index fluctuations) and a cell's dry mass density (i.e., nonaqueous content). To quantify the phase information about the specimen, the interference pattern produced by the superposition of the specimen and reference field is recorded, i.e., the phase information of the specimen is retained by the modulated intensity distribution. There are several QPI techniques that are used to quantify the information about the biological samples [5,6].

In most of the QPI techniques, a highly spatially and temporally coherent light source, such as a laser source, is used to acquire the interference pattern easily. However, there are some disadvantages of using highly coherent (i.e., long coherence length) light sources for QPI. Highly coherent light leads to the generation of unwanted speckle patterns and parasitic fringes from multiple layers of the object and from the optical components simultaneously, which degrades the image quality and reduces the phase sensitivity [6,7]. The signal-to-noise ratio (SNR) of the highly coherent laser-based QPI technique has been thoroughly studied in [8]. Furthermore, high brightness of laser light may also damage the delicate biological cells. To overcome these problems, partially coherent light sources having optimum power for the biological samples can be used. Temporally low-coherent (spectrally broad band) light, such as a white light source [9], super-luminescent diode (SLD), Ti:sapphire pulsed laser, and light-emitting device (LED) [10] has been used for QPI to reduce the unwanted speckle noise and eliminate the spurious fringes. However, there are certain disadvantages as well to use aforementioned spectrally broad band light sources in QPI, i.e., it requires dispersion compensation mechanism for the dispersion correction. Further, it is also not a good choice to use a spectrally broad light source if the object or medium has an inhomogeneous spectral response [11]. Therefore, the use of a spectrally narrow, i.e., monochromatic (temporally highly coherent) and spatially extended, i.e., spatially incoherent (pseudo-thermal) light source may have advantages over the spectrally broad band light source in these situations. There is no requirement of any dispersion compensation optical element for imaging the object having strong dispersion or inhomogeneous spectral absorption. The light sources having high temporal coherence (spectrally narrow) and low spatial coherence (wide angular spectrum) have been synthesized by many authors just by passing the laser light through the rotating diffuser [11–13] or stationary diffuser, followed by vibrating a multiple multimode fiber bundle (MMFB) [14]. Spatially low-coherent light obtained by aforementioned procedures or illuminating a speckle field [15] can also be helpful for the reduction of speckle noise significantly as in the broadband light sources.
In this Letter, we demonstrate the use of narrow temporal frequency and wide angular frequency spectrum light source for the measurement of phase and height profiles of biological samples, such as human RBCs and onion cells. The concept of spatial, angular, and temporal diversity was used to synthesize a low spatial coherent light source using a He–Ne laser light source. The main advantage of using temporally high and spatially low coherent light source for studying these parameters (phase and refractive index) of biological samples is that it does not require any dispersion compensation mechanism for dispersion correction, and no speckle or spurious fringes can be formed. Further, a very low-cost laser was used.

Different QPI techniques work on the principle of interferometry to measure the phase shift produced by the biological sample. The two-dimensional intensity modulation produced by the superposition of object and reference waves can be written as follows [16,17]:

\[ I_n(x, y) = I_R(x, y) + I_S(x, y) + 2\sqrt{I_R(x, y)I_S(x, y)} \cos(\Delta \phi + n\alpha), \]

where \( I_R \) and \( I_S \) are the intensities of the reference and sample beam, respectively; \( n \) (\( n = -2, -1, 0, 1, 2 \)) corresponds to the different phase-shifted interferograms; \( \Delta \phi \) is the phase difference between the sample and reference arm; and \( \alpha \) is the phase shift between the consecutive phase-shifted interference patterns for wavelength \( \lambda \). The phase difference between the sample (cells + medium) and reference arm can be given by the following expression [17]:

\[ \Delta \phi(x, y) = \tan^{-1}\left[\frac{2(I_1(x, y) - I_4(x, y))}{I_2(x, y) - 2I_0(x, y) + I_3(x, y)}\right]. \]

The phase map of the sample (cells + outside medium) “\( \varphi \)” can be obtained by subtracting the phase of the reference field from “\( \Delta \phi \).” The reconstructed phase maps can be utilized to determine the refractive index profiles or height maps of the biological cells using the following expression:

\[ \varphi(x, y) = \frac{2\pi}{\lambda} (n_{cell}(x, y) - n_{medium}(x, y)) \times 2h(x, y), \]

where \( n_{cell}(x, y) \) and \( n_{medium}(x, y) \) are the refractive indices of the cell and medium; \( h(x, y) \) is the cell thickness. Equation (3) can be rewritten as

\[ h(x, y) = \frac{\lambda}{4\pi\Delta n} \varphi(x, y), \]

where \( \Delta n \) is the refractive index difference between the cell and outside medium.

In this Letter, QPI of the human RBCs and onion cells was performed using He–Ne (wavelength 632 nm, 10 mW) laser. The study was conducted on the discarded blood samples taken from unknown persons and collected from the hospital. Ethical clearance from the IIT Delhi ethics committee has been taken for testing blood samples of a healthy person using this method. The blood samples were handled by an appropriate technical and trained person. Both the samples were prepared on the aluminum coated glass slide. Figure 1 shows the schematic diagram of the experimental setup. More details about the experimental setup are given in our previous paper [14]; however, the setup is briefly described here for the reader’s convenience. The light beam coming from the laser (temporal coherence length \( \approx 15 \text{ cm} \)) was made incident onto the beam splitter BS₁ and divided into three beams by means of MOs and multimode fiber Y-coupler, as shown in Fig. 1. The three diverging beams were incident onto the stationary diffuser from three different angles \( +40°, 0°, \) and \(-40° \), called angular diversity.

The laser beam at normal incidence (0°) and the two angular beams generate statistically independent (temporally incoherent) speckle patterns after getting scattered from the diffuser. They will add on the intensity basis and the speckle contrast is reduced significantly [18]. The scattered light from the diffuser containing a wide range of angular frequencies was coupled to a vibrating MMFB (hundreds of fibers of equal length, each fiber having core diameter 0.1 mm) by a condenser lens (focal length \( \sim 17.5 \text{ mm} \)). At the output of a vibrating MMFB, M, mutually spatially and temporally incoherent point sources are generated to produce M independent speckle patterns, which add on an intensity basis. Thus, a spatially low coherent (extended) light source is generated with the combined effect of spatial, angular, and temporal diversity. The spatially low coherent light at the output of a MMFB was coupled to the NIKON microscope (Nikon Eclipse 50i), which was finally incident onto the attached Mirau interferometer objective lens. The interference pattern was recorded by a CCD camera [Lumenera Infinity 2, 1392 x 1024 pixels, pixel size: 4.65 x 4.65 \( \mu \text{m} \)]. To record five phase-shifted interferograms, the Mirau interferometric objective lens is attached with a piezo controlled adapter (Piezo, Jena, MIPOS 3), which is driven by an amplifier, as shown in Fig. 1. Piezo moves the inbuilt reference mirror, as well as the imaging lens, toward the vertical direction. This introduces a phase shift corresponding to the change in OD of the reference and sample arms. The five \( \pi/2 \) phase-shifted interferograms are then recorded by a CCD camera. During the recording of all frames, samples were within the depth of field of the objective lens. All five frames were captured within 1 s using a color CCD camera of frame rate 10 fps. For the phase extraction of the specimen from these recorded frames, image post processing is done. The post processing took \( \sim 50–60 \text{ s} \). After synthesizing the spatially low coherent monochromatic light source, the longitudinal spatial coherence length of light source obtained was 9 \( \mu \text{m} \) while using the 10 x microscope objective (imaging) lens [14]. The effective coherence length is reduced due to the combined effects: (1) superposition of the three angular beams at the diffuser plane, (2) various path lengths within the MMFB, (3) mode coupling within the multimode fiber, and (4) numerical
aperture (NA) of the imaging lens. Obtaining such a short longitudinal coherence length leads to the elimination of spurious fringes in the laser based optical setups, and angular diversity leads to the reduction in the speckle contrast. Therefore, with these achievements a laser source can be used for the quantitative phase imaging of delicate biological cells, which is demonstrated in this Letter.

In all QPI techniques, phase noise is an important parameter, which tells about the stability of an interferometer. Therefore, first we measured the phase noise of the present setup. To measure the phase noise, a 1 min time lapse interferometric movie was recorded by placing a mirror on the sample stage under the stable environmental condition.

Phase noise of the setup is measured in all three different cases (single, double, and triple beam). The phase noise of the setup does not depend on the coherence length of the light source, i.e., angular multiplexing of the beam. It only depends on the stability of the interferometer. Therefore, phase noise of the system is found to be almost the same in all three cases. Even though the phase noise is approximately the same in all three different cases, angular multiplexing of three beams reduces the speckle contrast and eliminates the spurious fringe formation. Further, it does not require any dispersion compensation mechanism due to narrow spectral bandwidth of the laser light source. The phase noise of the setup is obtained less than 10 mrad, which corresponds to the 0.4 nm sensitivity in height measurement, as shown in Fig. 2. Once the phase noise of the present setup is measured, accuracy of phase measurement was calculated by performing the experiment on a standard strip waveguide chip made of a thick core layer (height $\sim 220 \pm 10$ nm) of tantalum pentoxide ($\mathrm{Ta_2O_5}$). $\mathrm{Ta_2O_5}$ has a refractive index value of around 2.1359 at an operating wavelength 632 nm. Figure 3(a) shows the height profile of the waveguide chip measured from a standard surface profilometer (P-7 stylus profiler). The height of the $\mathrm{Ta_2O_5}$ layer was found to be equal to 224.88 nm. Further, the experiment was performed on the same waveguide chip using the proposed technique shown in Fig. 1. Figure 3(b) shows one of the interferograms from the set of five phase-shifted interferograms. These five phase-shifted interferograms were further used to measure the phase, as well as the height profile of the waveguide chip. The phase and height map of the chip are calculated using Eqs. (3) and (4), as shown in Figs. 3(c) and 3(d), respectively. The height of the chip is found to be equal to 225.56 nm, which is very close to the value obtained using surface profilometer.

Next, the experiment was performed for the QPI of RBCs using a Mirau-interferometric objective of magnifications $50 \times$ (NA = 0.55). The $50 \times$ objective provides a sufficient field-of-view and required transverse resolution for imaging of human RBCs. A set of five phase-shifted interferograms are recorded with and without vibrating the MMFB for the reconstruction of the RBCs phase map, as shown in Fig. 4. Figures 4(a) and 4(b) clearly depict the difference in the interferometric image quality of RBCs without and with a vibrating MMFB. It can be seen that the image quality is quite bad in the case of static MMFB, i.e., no RBCs feature is visible whereas, a vibrating MMFB significantly improves the image contrast, as shown in Fig. 4(b). To reduce the speckle contrast, the MMFB is vibrated at a constant frequency $\sim 150$ Hz. For the reconstruction of the RBCs phase map, five equally $\pi/2$ phase-shifted interferograms were recorded by the CCD camera. The exact $\pi/2$ phase shift is introduced between the reference and sample arm with the help of piezo controlled objective lens adapter.

Figures 4(a) and 4(b) show one of the interferograms from the five phase-shifted interferograms without and with a vibrating MMFB, respectively. From five phase-shifted interferograms, the wrapped phase maps were obtained using the five-frame phase-shifting algorithm given in Eq. (2). The minimum $L^2$-norm two-dimensional phase unwrapping algorithm

![Fig. 3.](image-url) (a) Height map of a waveguide chip measured from surface profilometer, (b) interferogram, (c) unwrapped phase map, and (d) height map of the same chip using the present setup at red wavelength.

![Fig. 4.](image-url) (a), (b) Interferograms and (c), (d) corresponding phase map of Human RBCs, without and with vibrating MMFB, respectively.
was further used to unwrap the wrapped phase map of sample. Figures 4(c) and 4(d) show the unwrapped phase maps of RBCs (image size ∼48 μm × 60 μm) at 632 nm wavelength. In Fig. 4(d), there are a few artifacts present in the background of the RBCs phase image. These artifacts arose due to the error in the phase shift between the two consecutive phase-shifted interferograms generated by either the piezo stage or environmental fluctuation. Ideally, phase shift between the two consecutive frames must be equal to $\pi/2$ or a constant number “a” for the artifacts’ free phase images. If, somehow, the piezo stage does not employ the exact phase shift between the two consecutive frames, then this kind of artifact can be present in the reconstructed phase images. It is clear from the phase maps; phase information cannot be retrieved about the biological cells in the case of static MMFB, which is completely filled with the speckle noise. The speckle noise is reduced with a vibrating MMFB due to the temporal averaging of the speckle patterns. Therefore, it is good to use a vibrating MMFB for the QPI of biological cells. In addition, it would be a good practice, in general, to get the speckle-free images by employing a vibrating MMFB. The maximum phase value of the RBCs is found to be 3.042 rad with a vibrating MMFB.

Similarly, the experiment was performed on onion cells for the QPI at 632 nm wavelength with a vibrating MMFB using a $10 \times$ (NA = 0.33) microscope objective lens.

Since the size of onion cells are bigger than RBCs, the $10 \times$ objective provides a sufficient field of view and required transverse resolution for imaging onion cells. Figure 5(a) shows one of the interferograms from the set of five phase-shifted recorded interferograms at 632 nm wavelength. The unwrapped phase maps for the onion layer were obtained by following a procedure similar to the used for RBCs, as shown in Fig. 5(b). It is clear from the phase map that the onion skin is not uniform due to different thicknesses of the cells at different positions. Therefore, the OPD is also different at each position. The height map of onion cells was obtained by using the value of $\Delta n$ equal to 0.3345 for red wavelength. This value was obtained at the refractive index values of onion layer and outside medium for red wavelength equal to 1.3345 and 1.0, respectively [20]. Figures 5(c) and 5(d) show the corresponding height map and line profile of a few onion cells, respectively. The maximum height of the cell is found to be ∼5 μm at the center of the cell.

In summary, the quantitative phase imaging of biological cells (RBCs and onion cells) using a low spatial coherence light source is demonstrated. The present method is a potential technique for the quantitative phase imaging of biological samples at 632 nm wavelength with high phase sensitivity of 10 mrad that corresponds to a very small fraction of the optical wavelength. Using the present system from the phase-shifted interferograms, the phase and the height map of the biological cells were obtained without using any dispersion compensation mechanism. The possible advantage of the present technique is the good contrast of the interference fringes because it does not suffer from the problem of cross-talk due to larger spectral width of Bayer color filters, as compared to the broad band light sources. Further, it uses a low spatial and high temporal coherence light source and, hence, imaging is speckle free, and no spurious fringes are formed. The present technique is useful for obtaining the morphological information about the cells and tissues without damaging them.

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