Quantitative phase-contrast confocal microscope

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Abstract We present a quantitative phase-contrast confocal microscope (QPCCM) by combining a line-scanning confocal system with digital holography (DH). This combination can merge the merits of these two different imaging modalities. High-contrast intensity images with low coherent noise, and the optical sectioning capability are made available due to the confocality. Phase profiles of the samples become accessible thanks to DH. QPCCM is able to quantitatively measure the phase variations of optical sections of the opaque samples and has the potential to take high-quality intensity and phase images of non-opaque samples such as many biological samples. Because each line scan is recorded by a hologram that may contain the optical aberrations of the system, it opens avenues for a variety of numerical aberration compensation methods and development of full digital adaptive optics confocal system to emulate current hardware-based adaptive optics system for biomedical imaging, especially ophthalmic imaging. Preliminary experiments with a microscope objective of NA 0.65 and 40 × on opaque samples are presented to demonstrate this idea. The measured lateral and axial resolutions of the intensity images from the current system are ~0.64 μm and ~2.70 μm respectively. The noise level of the phase profile by QPCCM is ~2.4 nm which is better than the result by DH.

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1. Introduction

Point-scanning confocal microscopy was originated by M. Minsky in 1961 to obtain high-resolution intensity images and optical sectioning of the samples [1]. It has proven to be successful for noninvasive imaging of thin sections within thick biological samples with high resolution and contrast [2, 3]. It has also been widely applied in industrial inspection [4, 5]. However, the speed of the image acquisition is limited by the point-scanning configuration. To speed up image acquisition and simplify the optical system, line-scanning confocal systems have been proposed and tested in industrial inspection, imaging of human tissues, and ophthalmology [6–9]. Equipped with adaptive optics, the line-scanning confocal ophthalmoscope is able to image the human retina at the cellular level [10]. Instead of scanning one point in the object at a time, one line is scanned at a time. This scanning scheme with a linear charge-coupled device (CCD) has gained more and more attention because it is fundamentally simpler and faster compared to the point-scanning confocal system. More importantly, the lateral and axial resolutions of the biological images are comparable with the point-scanning system [7–10]. Similar to the point-scanning confocal system, the line-scanning confocal system is unable to get the quantitative phase information of the optical field that is of great interest in industrial inspection and biomedical imaging.

On the other hand, DH is able to get access to the complex amplitude of the optical field from which quantitative phase information can be retrieved [11, 12]. This feature finds DH wide applications in many fields such as industrial inspection, biological imaging, adaptive optics and so forth [13–15]. In most cases, a coherent light source is necessary to perform the DH experiment. Inherent in this coherent imaging modality, speckle noise is still an issue that has severely limited the application of DH in imaging scattering samples such as human tissues. Another limitation of the DH is its lack of optical sectioning capability.

The first effort of combining the confocality with off-axis DH was made in 2012 [16], and confocal phase maps of biological cells were reported in a follow-up paper in 2013 by the same group [17]. In these original proposals, a point-scanning system was adopted. For each point of the sample, a hologram is recorded and numerically processed. The amount of data involved to reconstruct a full-field image is at the level of Terabytes. This huge data flow will make this original scheme hard to find practical applications in industrial testing and biomedical imaging in the near future. To simplify the optical system and speed up the data acquisition and processing, we explored the possibility of combining a line-scanning confocal configuration with off-axis DH. The presented imaging system QPCCM can take high-quality intensity images of optical sections and provide quantitative phase map at each optical section at a speed that is at least three orders of magnitude faster than the original digital point-scanning confocal system. The data involved can be easily handled by a regular desktop computer. In our experimental setup, the CCD is put at the image plane of the sample instead of Fourier plane as adopted by the original digital confocal microscope. The whole optical field of the sample is reconstructed without a need of performing numerical propagation. Also, a stronger signal is collected with the CCD at the image plane for imaging weakly
scattering object. The potential applications of QPCCM in industrial inspection and biomedical imaging are believed to be immediate. Since each line scan records all the information of one slice of the object including the aberrations of the system, it opens the avenues for a variety of numerical aberration compensation methods and development of a full digital adaptive optics system for biomedical imaging especially ophthalmic imaging [14, 15, 18, 19].

The paper is organized as follows: The optical system of QPCCM is described in section 2. In section 3, the experimental results are presented and discussed. Finally, the conclusions are drawn.

2. Optical system

The schematic diagram of QPCCM system is illustrated by Fig. 1. Figure 1(a) shows the top view of the optical setup. He-Ne laser is the light source with a wavelength of 632.8nm. The laser beam collimated by the beam expander BE1 is sent to a cylindrical lens CL with a focal length of 75mm and forms a diffraction-limited focal line at the back focal plane of the microscope objective MO (NA 0.65, 40 × ). This illumination configuration is unfolded in Figs. 1(b) and 1(c). The coordinates of the optical system are shown to the right of Fig. 1(b). Figure 1(b) shows the illumination in the xz plane where the light is focused at the back focal plane of the CL and front focal plane of the MO and a collimated line is generated on the sample S in the x direction (horizontally). The illumination in the yz plane is shown in Fig. 1(a), where the light is focused at the back focal plane of the MO. As a result, a focal line is formed in the x direction at the sample. The CCD with 1024 × 768 square pixels of a side length 4.65μm is put at the conjugate plane of the sample S. In the experiment, an area of interest with 512 × 512 pixels is used to speed up the data acquisition and processing. The calibrated magnification between the CCD and object planes is 43.5. The sample S is mounted on a motorized translation stage. The CCD is triggered by a data acquisition device (Labjack, U3-LV) at the rate of 20 frames/s. The sample is continuously moved in the y direction (vertically) at the speed of 2.14μm/s during the image acquisition so that the pixel resolutions in both the scanning and non-scanning directions are consistent and also satisfy the Nyquist sampling requirement. To generate an off-axis hologram for each line scan, a laser beam collimated by the beam expander BE3 is introduced and reaches the CCD at a small angle with respect to the light from the sample [11–13]. The exposure time for each hologram is set to be ~0.5ms to remove the motion blurring. To facilitate the adjustment of the sample position and compare the result of QPCCM with that of DH, we introduce a third laser beam indicated by the arrowed lines through the beam expander BE2 and the regular lens L2 in Fig. 1(a) to obtain wide-field microscopic images and holograms.

To build up a full-field image at one optical section, a video of 512 holograms is recorded and processed by Matlab 2008b on a Dell desktop computer [Intel(R) Core(TM Duo CPU, 4 Gigabytes memory)] to reconstruct the intensity and phase images. It takes ~26 seconds to complete the data acquisition and ~2 minutes to reconstruct the intensity and phase images of an optical section with 512 × 512 pixels. It is worth noting that no physical slit aperture is added in the optical system. A numerical slit is applied in the numerical reconstruction. The basic process of image reconstruction will be demonstrated in subsection 3.1.
3. Experimental results and discussions

3.1 Basic process of image reconstruction

To demonstrate the basic process of the confocal image reconstructions, a negative 1951 United States Air Force (USAF) resolution target is used as the sample. The hologram of one scan is shown in Fig. 2(a). The detailed view of the region in the white square in Fig. 2(a) is shown in Fig. 2(b) where the interference fringes are displayed. The angular spectrum of the hologram in Fig. 2(a) is shown in Fig. 2(c) in logarithmic intensity scale. The region indicated by the white circle is extracted and used to reconstruct this slice of the sample [13]. The resultant intensity $I_n(x,y)$ and phase map $\Phi_n(x,y)$ are shown in Figs. 2(d) and 2(e) respectively, where $n$ indicates the nth scan and the phase map is displayed in blue-white-red color map (same for all the phase maps in the remainder of this article). The confocal intensity $I_{conf}(x,n)$ of this scan is obtained by summing intensity values of $I_n(x,y)$ within a numerical slit along y direction, as follows

$$I_{conf}(x,n) = \sum_{y \in \text{slit}} I_n(x,y)$$

(1)

where the slit means the applied numerical slit indicated by the green rectangle in Fig. 2(d). The slit width $S_w$ is determined by one diffraction-limited resolution element, which is given by

$$S_w = \frac{0.61 \lambda M}{P \times NA}$$

(2)
where $\lambda$ is the wavelength of the light source, $M$ is the magnification of this imaging system, $NA$ is the numerical aperture of the MO and $P$ is the pixel size of the CCD. The result calculated by this equation is 5.55 pixels. We set $S_w$ to be 5 pixels. In fact, the slight change in the slit width bears negligible effect on the reconstructions. The full-field confocal intensity image is obtained by stitching together 512 confocal intensity lines given by Eq. (1). The reconstructed full-field intensity image is shown in Fig. 2(f). Compared to the wide-field image illustrated in Fig. 2(g), the confocal intensity image clearly shows higher contrast and lower coherent artifact.

The confocal phase profile $\Phi_{conf}(x,n)$ of each scan is obtained by taking average of the phase values of $\Phi_n(x,y)$ within the numerical slit along $y$ direction, as follows

$$
\Phi_{conf}(x,n) = \frac{\sum_{y\in S} \Phi_n(x,y)}{S_w}
$$

(3)

The reconstructed full-field confocal phase map is shown in Fig. 2(h). Random phase shifts among different line holograms due to the mechanical vibrations prevent a two-
dimensional phase map from being visualized. These phase shifts can be removed by the following numerical procedures:

Step 1, Subtracting the phase values in the nth row from those in the (n-1)th row in a pixel-wise way;

Step 2, Picking the value with maximum likelihood as the phase shift and correcting the nth row by subtracting this phase shift from it and wrapping the result into the range \((-\pi, \pi]\);

Step 3, Increasing n by one and repeating steps 1 and 2 until last row.

Note that in the first step the (n-1)th row has already been corrected. The corrected phase map is shown in Fig. 2(i). This procedure is based on the observations that the neighboring line phase profiles have similar shapes and that phase shifts across them are the same.

3.2 Measurements of system resolutions

The edge spread functions (ESF) can be used to test the lateral resolutions of intensity images [5]. The standard way is imaging a sharp edge object. In our experiment, an edge from a Ronchi ruling (20lp/mm) is imaged. Figure 3(a) shows the ESF in the non-scanning direction (x direction). The 20%-80% width as indicated by the distance of the two vertical dashed lines in Fig. 3(a) is used to estimate the lateral resolution in this direction that is \(\sim 0.64\mu m\). The ESF in the scanning direction (y direction) is shown in Fig. 3(b). This curve shows a smoother boundary at the edge than the ESF in the non-scanning direction because of the confocality. The 20%-80% width as measured by the distance of the two vertical dashed lines in Fig. 3(b) is also \(\sim 0.64\mu m\). These estimates of the lateral resolutions are close to the diffraction-limited resolution that is \(0.59\mu m\) and can be verified by the confocal intensity image shown in Fig. 2(f) where the width of the smallest bar is 2.14\mu m. 0.64\mu m is a quite close estimate of the actual resolution. The lateral resolutions of the phase images are close to those of the intensity images as evidenced by the phase map in Fig. 2(i). The axial resolution of intensity images can be tested by measuring the power within the numerical slit of the images of a mirror while it is moved through the focal plane [20]. The axial response with respect to the axial distance away from the focal plane is given by Fig. 3(c). The axial resolution can be estimated by the full width at half maximum (FWHM) of this curve that is \(\sim 2.70\mu m\), as indicated by the distance of the two vertical dashed lines in Fig. 3(c). The accuracy of phase map at each optical section will be discussed in subsection 3.3.

![Fig. 3. Measurements of lateral and axial resolutions. (a) Edge spread function in x direction. (b) Edge spread function in y direction. (c) Axial response with respect to the axial distance away from the focal plane.](image)

3.3 Confocal phase map

A phase object is made by depositing a layer of chrome on top of a positive 1951 USAF resolution target to remove the amplitude contrast. The height of the bars on the target is around 100nm that is well within one axial resolution element [21]. Thus, both the top and bottom planes are in focus. The phase map obtained by QPCCM is shown in Fig. 4(a). The
The relationship between the height and the phase is given by

$$\text{Height} = \frac{\text{Phase}}{4\pi \lambda} \quad (4)$$

where $\lambda$ is the wavelength of the laser. The denominator is $4\pi$ instead of $2\pi$ because the imaging system is in reflection mode. The height of this cross section is calculated as 100.8nm. The noise level can be visualized by the height profile of a cross section through an empty region, as shown in Fig. 4(c) that is the height profile of the cross section indicated by the dashed line in Fig. 4(a). The noise level is measured by evaluating the standard deviation of a flat region indicated by the dashed square in Fig. 4(a) that is calculated as 2.4nm. For comparison, DH is performed on the same area of the target. The phase map obtained by DH is shown in Fig. 4(d). The height profile at the cross section indicated by the solid line in Fig. 4(d) is shown in Fig. 4(e). The height of this cross section is calculated as 105.0 nm. Figure 4(f) shows the height profile of the cross section indicated by the dashed line in Fig. 4(d). Compared to Fig. 4(c), it shows stronger height variation, which means the noise level of DH is worse than the QPCCM. By evaluating the standard deviation of a flat region indicated by the dashed square in (d), the noise level is calculated as 4.8nm. A more intuitive comparison of the noise levels of Figs. 4(a) and 4(d) can be given by Figs. 4(g) and 4(h). It is quite obvious that the phase image of Fig. 4(h) is smoother than that of Fig. 4(g), indicating the noise level of QPCCM is better than DH.
The effect of the slit width on the phase profile is investigated by observing how the phase profile of the cross section in Fig. 4(b) changes as the slit width. By convention, the unit of slit width adopted here is in Airy Unit (A.U.) that is given by [4]

$$AU = \frac{1.22\lambda}{NA}$$

One A.U. is the diameter of the first dark ring of the Airy pattern. As illustrated by Fig. 5(a), when the slit width is within several A.U., the phase profiles do not change much. After several A.U., the phase profiles start deviating away from the normal phase profiles and finally lose the phase information as the slit width becomes too large. This process can be more clearly monitored by the change in the measured height as the slit width, as shown in Fig. 5(b). When slit width is within about 2 A.U., the measured height stays almost the same. As the numerical slit increases, the strong phase fluctuations outside the focal line will come into play and finally destroy the phase information when the slit is too large. It can be seen that, after about 2 A.U., the measured height begins decreasing and finally becomes meaningless when slit width becomes too large. This observation indicates that the phase map of QPCCM is not sensitive to the slit width when it is within about 2 A.U..

3.4 Optical sectioning

In subsection 3.1, we have demonstrated that the intensity image of QPCCM is better than the wide-field coherent image in terms of the contrast and coherent noise. The experimental results in subsection 3.3 indicate that QPCCM can get an even better phase map than DH. Another important characteristic of QPCCM is its capability of optical sectioning. In fact, we have measured its axial resolution that is ~2.70μm in subsection 3.2. In this subsection, we will demonstrate this capability by imaging a silicon wafer at different depths. We will also demonstrate that the phase maps at different depths can be obtained. The silicon wafer is made by photolithography and the average depth of the patterns is about 20.1μm, which is obtained by an optical profiler (Veeco Instruments Inc.). Figs. 6(a)-6(c) show the wide-field laser images at three different axial distances z = 0μm, 10μm, and 20μm respectively. It is apparent there is no optical sectioning for wide-field imaging. Figures 6(d)-6(f) show the confocal intensity images at these three depths by QPCCM. At z = 0μm, the top layer of the silicon wafer is focused and other parts of the image become dark. At z = 10μm, no apparent plane is focused. When z is set to be 20μm, the bottom layer of the etched lines are focused and other parts of the image become dark. To further demonstrate the optical sectioning, a confocal xz section at the position indicated by the dashed in Fig. 6(f) is illustrated by Fig. 6(g) where one can discern the bottom and top layers. The depth of the left hole can be measured as the difference in z values that correspond to the maximum intensities of the top
and bottom surfaces of the left hole in Fig. 6(g), which is ~20.7 μm. Similar to the conventional line-scanning confocal microscope, the numerical slit plays a role in enabling the optical sectioning. If the numerical slit is not applied, the capability of optical sectioning will disappear. It is worth noting that removal of the numerical slit means extending the numerical slit to the whole image height. Figures 6(h)-6(j) are the intensity images at the three depths when the numerical slit is removed. We cannot see any characteristics of the optical sectioning. The loss of optical sectioning because of removal of the numerical slit is clearly illustrated by Fig. 6(k) that shows the non-confocal counterpart of Fig. 6(g). In this image, the layered structure is totally lost.

Different from the conventional line-scanning confocal microscope, QPCCM is able to get the quantitative phase maps of the confocal planes. This characteristic can be illustrated by the confocal phase maps shown in Figs. 6(l)-6(n). When the top layer of sample is focused, we can measure the height variation of this focused surface as shown in Fig. 6(l). At \( z = 10 \mu m \), there is no apparent focal plane, therefore the corresponding phase map shown in Fig. 6(m) is of no practical interest. What is of interest is the phase map at the bottom layer as shown in Fig. 6(n), which may reflect irregularity of the etched surfaces. There are two strips in the phase maps that correspond to the bright regions in Fig. 6(f). These two pieces of phase maps can provide us with a quantitative way to assess the height variations of the bottom layer.

Fig. 6. Confocal intensity images and phase maps of optical sections of a silicon wafer. (a)-(c) Wide-field images at \( z = 0 \mu m, 10 \mu m \), and \( 20 \mu m \). (d)-(f) Confocal intensity images at \( z = 0 \mu m, 10 \mu m \), and \( 20 \mu m \). (g) Confocal xz section at the position in xy plane indicated by the dashed line in (f). (h)-(j) Scanning images without numerical slit at \( z = 0 \mu m, 10 \mu m \), and \( 20 \mu m \). (k) Non-confocal counterpart of (g). (l)-(n) Confocal phase maps at \( z = 0 \mu m, 10 \mu m \), and \( 20 \mu m \). Scale bars in (a) and (g): 10 μm.
Conclusions

We have experimentally demonstrated a quantitative phase-contrast confocal microscope that is fundamentally faster and simpler compared to the point-scanning digital confocal system [16, 17]. QPCCM can obtain the quantitative phase profiles at an even better noise level than DH. Optical sectioning capability and high-contrast intensity imaging with low coherent noise promise its potential applications in industrial inspection and biomedical imaging. QPCCM also opens the avenues for a variety of numerical compensation methods and development of a full digital adaptive optics system for biomedical imaging especially ophthalmic imaging [14, 15, 18, 19].

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