Quantitative phase microscopy with asynchronous digital holography

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Abstract

We demonstrate a new method of measuring quantitative phase in imaging of biological materials. This method, asynchronous digital holography, employs knowledge of a moving fringe created by acousto-optic modulators to execute phase-shifting interferometry using two near-simultaneous interferograms. The method can be used to obtain quantitative phase images of dynamic biological samples on millisecond time scales. We present results on a standard sample, and on live cell samples.

1. Introduction

Quantitative phase measurements have merited great interest lately for measuring cellular dynamics [1,2]. One natural modality for studying these dynamics is digital holographic interferometry (DHI) [3], a simple interferometric configuration that can be used to assess subwavelength changes in an object by measuring the optical path length difference between each imaged point at two distinct times. There are complications, however, in utilizing DHI at a high enough speed to visualize cell dynamics, usually considered to be ~10 ms. Primarily, unambiguous phase extraction requires separating the phase information from the rest of the signal in the interferogram. In this paper, we present a system designed to overcome these complications. The system uses asynchronous digital holography (ADH), a phase referencing method based on acousto-optically generated moving fringes, to perform quantitative phase microscopy. We show that ADH obtains quantitative phase images on the 10 ms time scale, and demonstrate its application to several biological systems.

The goal of holography-based phase imaging is to extract the phase from a holographic image. To do this, the method may take one of three approaches: First, multiple images can be recorded to gain enough information to yield the phase term, second, the background can be removed via optical processing, or third, the background can be removed through digital image processing. Zhang and Yamaguchi [4] use the first approach, implementing a phase-shifting DHI scheme that combines four interferograms to yield the phase term. This works well with stationary samples, but does not work well with samples that change on millisecond time scales, as considerable changes can occur in the time it takes to record four interferograms with a CCD. Additionally, the longer the recording time, the greater influence phase perturbations due to environmental disturbances have on phase image quality. Depeursinge and coworkers take the second approach, employing an off-axis digital holography system [5] that uses only one interferogram to reconstruct the phase information, thereby making it an imaging technique applicable to visualizing living cells [1]. In this method, the reference beam is tilted to induce spatial separation of the background from the desired signal. The tilt of the reference beam can

limit the field of view and spatial resolution of the phase image [6]. Feld and coworkers take the third approach; they created a system that uses only one image, isolating the phase term via image processing [7], similar to the Fourier transform evaluation method [8]. This system can be used for wide-field imaging and has no limitations on the spatial resolution; it has been successfully used to quantify cell structure and dynamics [2,9]. One possible disadvantage of this approach, among others [3], is that in using image processing to eliminate unwanted background, some desired signal may be eliminated as well.

We now propose an alternative quantitative phase microscopy method, asynchronous digital hologray (ADH) that integrates two new features into a holographic imaging setup. The first is the use of phase referenced interferometry. Phase shifting is achieved using acousto-optic modulators (AOMs), as proposed by Li [10]. Here the phase shift is measured, as opposed to tightly controlling it, to greatly simplify the experimental setup by eliminating the need for control electronics. The phase reference is obtained by tilting the reference beam slightly off-axis to observe several fringes across the image plane. These fringes, which move due to a frequency offset between the two AOMs, are analyzed to measure the phase-shift between successive images. The second feature is that only two images, which can be recorded in a near-simultaneous manner (as described in section 2), are used to find the phase distribution. This not only avoids the disadvantages of phase-shifting interferometry described above, but also permits us to eliminate the background and extract the phase unambiguously, without using image processing or introducing a large angular tilt in the reference arm. In the following sections, we will describe the ADH setup and processing algorithm and present quantitative phase microscopy images of water and living cells to demonstrate quantitative phase imaging of biological samples.

2. Experimental setup

Our experimental setup (Fig. 1) is similar to an off-axis digital holographic microscopy setup [11] with two modifications. The first is the use of a 4f optical configuration, with the CCD placed in the image plane. The second is inclusion of an AOM in each arm of the interferometer, allowing independent frequency modulation. For our experiments, the AOMs (1206C, Isomet, Springfield, VA) were set to 80 and $80 + \Delta$ MHz to yield a $\Delta = 100$ Hz beat frequency between them. The AOMs are synchronized by a frequency synthesizer (iDDS-2, Isomet), which provides excellent phase stability (0.004%) and control over the frequency offset. The frequency offset of 100 Hz was chosen so that the fringes move slowly enough that blurring due to the finite exposure time of the CCD is not a significant effect. The microscope objective (MO) is a 40x Zeiss Neo-Fluar, set up in a 4f configuration with a 150 mm focal length lens, L2, yielding a magnification of 33x. The reference arm beam is expanded by 20x using a 7.5 mm lens (L1) that is also in a 4f configuration with L2. A HeNe laser (λ=633 nm, Uniphase, Milpitas, CA) is the light source for the system.

The ADH system can record two images in a near-simultaneous manner using a frame transfer CCD that allows two successive images to be recorded on a microsecond time scale. We use the Photometrics Cascade:650 CCD in this setup. This camera can transfer a full frame (653×492 pixels) in 210 μs and has a 36.2 ms readout time. A reduced region of interest, for example a 300×200 pixel image, can be transferred in just 40 μs, enabling the recording of two images just 40 μs apart. When using frame transfer, both images can then be read out in 13.5 ms and used to find the quantitative phase distribution. In this way, quantitative phase measurements can be recorded at 74 frames/sec. In the work presented here, we did not employ this capability since we did not utilize dynamics fast enough to necessitate it.

3. Theory

There are two parts to a holographic image: the background and the part of the image that describes the phase relationship between the two beams. This can be written as

$$I(x, y)=A(x, y)+C(x, y)+C^*(x, y);$$
$$C(x, y)=\frac{1}{2}B(x, y)\exp[i\Delta\varphi(x, y)]$$

(1)

where x and y are CCD coordinates (i.e. pixel locations), A represents the background, B the amplitude modulation term, and $\Delta\varphi$ describes the phase difference at a particular point between the reference and sample beams. None of these terms are known a priori.

The angular offset of the two beams (reference and sample) causes a spatial carrier frequency, resulting in fringes. The AOMs impart a phase shift from one image to the next, thereby causing the fringe to move in time. The moving fringes are linear, due to the intrinsic wavefront matching in the 4f optical configuration. The fringe in each image is fit to a sine wave and analyzed to find both the phase shift between images, α, and the spatial frequency, q. Once the phase shift is known, the phase can then be found unambiguously:

$$I_1=A(x, y)+C(x, y)\exp(iqx)+c.c.;$$
$$I_2=A(x, y)+C(x, y)\exp\{i[qx+\alpha]\}+cc;$$
$$G=HT\{I_1 - I_2\}\exp(-iqx)/(1 - \exp(i\alpha);$$
$$\Delta\varphi(x, y)=\tan^{-1}\left(\frac{\mathrm{Im}G}{\mathrm{Re}G}\right).$$

(2)

In Eq. (2), I_1 and I_2 represent successive images with a phase shift α between them. These images are processed using a Hilbert transformation (HT) [12] and knowledge of the phase shift, α, to yield a quantitative phase image. In this algorithm, the subtraction of the two interferograms eliminates the term, A, thereby removing all common noise elements and background effects. This obviates the need for the low-pass filter often used in the Fourier transform evaluation method. The algorithm is summarized in Fig. 2.

The algorithm is demonstrated in Fig. 3. Two successive interferograms of part of a water droplet are shown in Figs. 3(a) and 3(b). A sine wave is fit to the same section of each interferogram, which is a section of the interferogram that is not affected by the water drop. From the sine wave fit, the spatial frequency, q, and the phase-shift, α, are determined. G and subsequently $\Delta\varphi$ are then computed as described in Eq. (2), with the result shown in Fig. 3(c), which is the wrapped phase. The unwrapped phase is shown in Fig. 3(d).

4. Results

The ADH system was initially tested with a water sample to determine the phase stability. The sample was 45×100 pixels so that we would have 200 interferograms in 1.0 second. The diffraction-limited resolution of the system is 0.63 μm, corresponding to a 3×3 pixel area. The average phase was found over a 3×3 region of the image for 100 phase images spread evenly over 1.0 second. With the processing method described above, in which two interferograms are used to reconstruct the phase distribution, the phase stability was found to be 4.9 nm (Fig. 4) by calculating the standard deviation of the data. This is of the same order as the phase stability reported for previously existing systems [7].

The capacity of the ADH system for measuring phase is first demonstrated on the water droplet depicted in Fig. 3. The results are shown in Fig. 4. For each quantitative phase image depicted

in Fig. 4, two interferograms were recorded 13.5 ms apart (without using frame transfer) and processed by Eq. (2); each image represents the quantitative phase of the water droplet at 1.0 second intervals. The images clearly show the recession of the water droplet due to evaporation. The maximum thickness of the water droplet (calculated from the difference of the phase at (1,1) and (80,134), i.e. the top-left and bottom-right corners of the phase map, respectively) is (a) 8.34 μm, (b) 6.34 μm, (c) 5.75 μm, and (d) 3.67 μm.

In Fig. 5, we present quantitative phase images of a red blood cell, left, and a smooth muscle cell, right. These quantitative phase images were unwrapped using standard phase algorithms presented in [13]. Here the interferograms were recorded 6 ms apart.

5. Discussion

An essential requirement that must be met to apply a phase microscopy system to imaging the dynamics of live cells is that the system can acquire quantitative phase images of the sample at a high rate (>100 Hz). Although modern CCDs are capable of 100 Hz image acquisition rates, multiple interferograms are often necessary to extract the phase information (see section 1 for exceptions and their limitations), which reduces the acquisition rate considerably. Additionally, if multiple inteferograms are used, they must be recorded quickly enough so that instabilities in the system and the dynamics of the cells themselves do not vary appreciably during acquisition.

Here we have demonstrated that our ADH system is capable of obtaining quantitative phase measurements on millisecond time scales. The inclusion of AOMs in each arm of the interferometer permits us to use phase-shifting interferometry. The system is innovative in the field of digital holography in that the phase shift is easily evaluated as opposed to controlled, which greatly simplifies the experimental setup. In addition, the ADH algorithm requires only two phase-shifted interferograms, compared to the usual 4 required in most phase shifting algorithms. A potential increase in speed can be realized by utilizing frame transfer CCD devices, which can record two images on a microsecond time scale. By transferring frames without reading them out, the latency between two interferograms is greatly reduced and the quantitative phase imaging frame rate is then nearly the frame rate of the camera, on the order of 100 Hz.

We have demonstrated, with a red blood cell sample, and a smooth muscle cell sample, that this system is capable of obtaining quantitative phase images of live cells. Future work will focus on making biologically relevant measurements of cell dynamics on millisecond time scales.

Acknowledgments

References and Links

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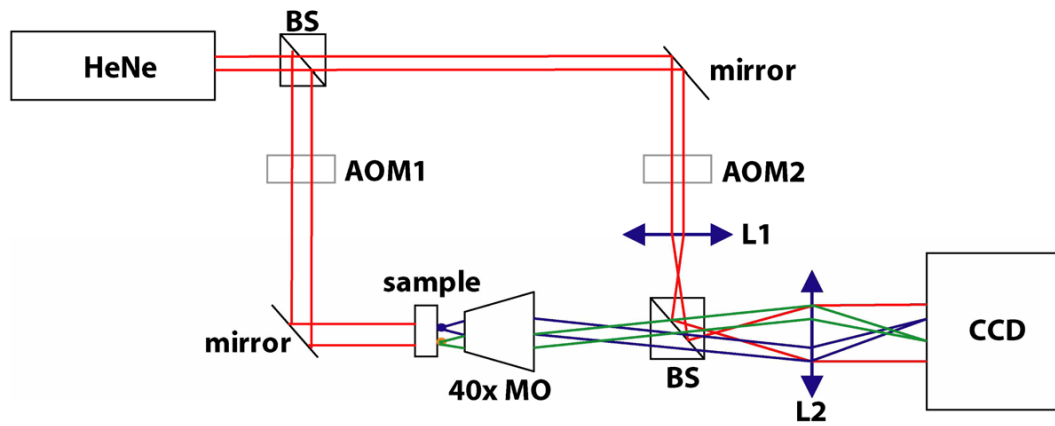
Fig. 1.
Setup for ADH system. The optical phase is tightly controlled with the AOMs (acousto-optical modulators). The MO and L1 form a 4f imaging system with L2, respectively, so that the CCD is in the image plane. The magnification of the sample beam is 33x, while the reference beam is expanded by a factor of 20.
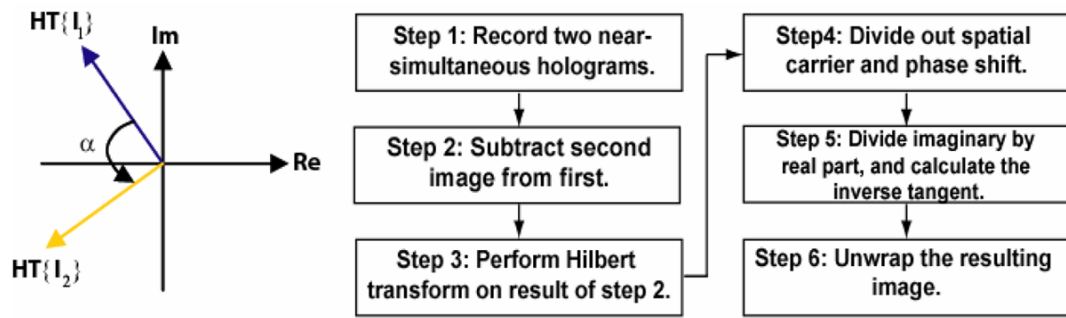
Fig. 2.
At left, the complex analytic signal after the Hilbert transform. The AOMs impart a known phase shift α on the signal. At right, a flow chart of the ADH algorithm.
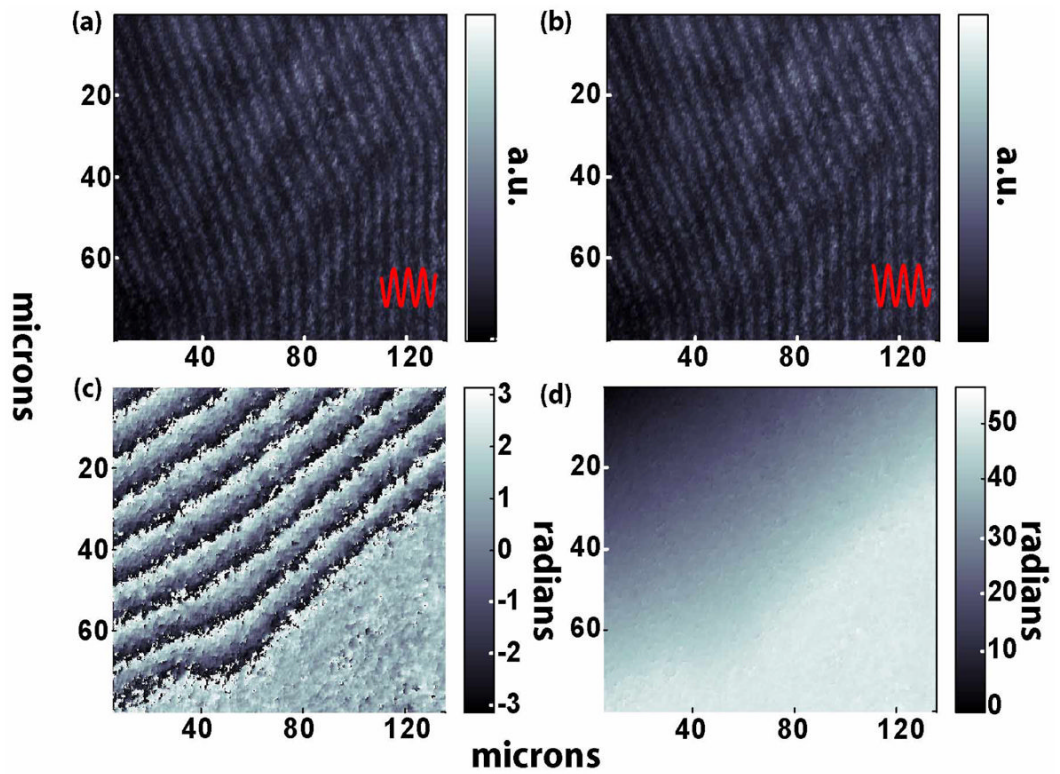
Fig. 3.
(a) and (b) depict two successive interferograms of the bottom-right quadrant of a water drop. A sine-wave is fit to the same section of each interferogram, and the wrapped phase (c) is determined from Eq. (3). The unwrapped phase is shown in (d).
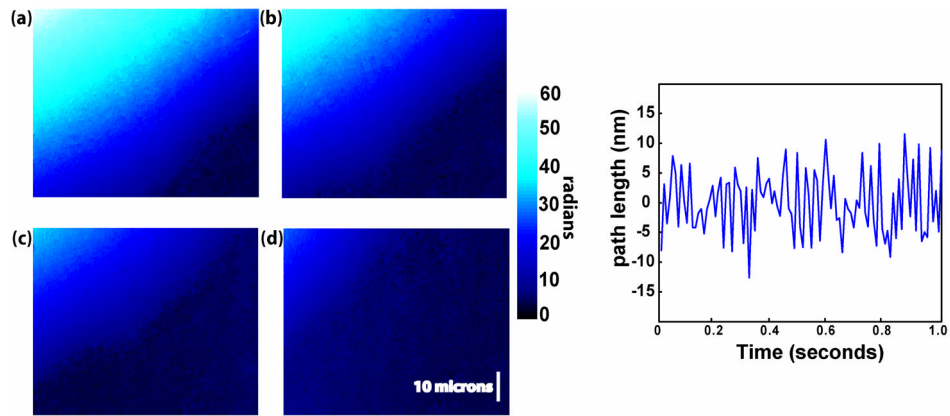
Fig. 4.
At left, Four successive quantitative phase images of a water droplet receding due to evaporation. Each image was recorded 1 second apart. The maximum thickness of the water droplet in the image is (a) 8.34 μm, (b) 6.34 μm, (c) 5.75 μm, and (d) 3.67 μm. At right is a time profile of the phase stability over a 3×3 pixel area of a water sample. The standard deviation of the phase is 4.9 nm.
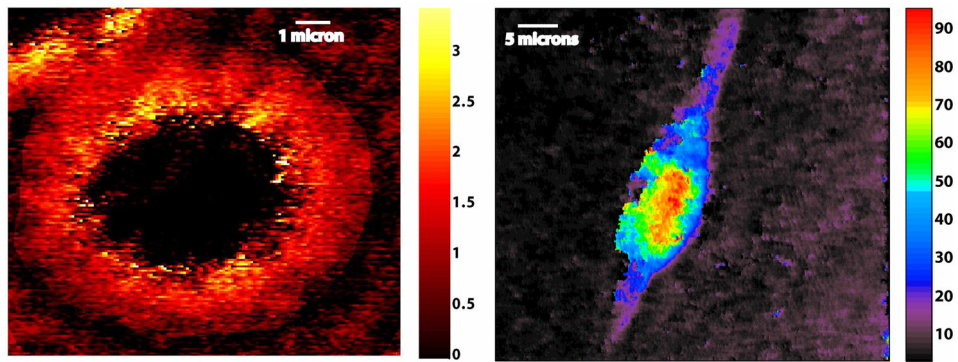
Fig. 5.
A quantitative phase image of a red blood cell (left) and a smooth muscle cell (right).