Dual-wavelength linear regression phase unwrapping in three-dimensional microscopic images of cancer cells

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We present a study of the three-dimensional structure of cancer cells using dual-wavelength phase-imaging digital holographic microscopy. Phase imaging of objects with optical height variation greater than the wavelength of light is ambiguous and causes phase wrapping. By comparing two phase images recorded at different wavelengths, the images can be accurately unwrapped. The unwrapping method is computationally fast and straightforward, and it can process complex topologies. Additionally, the limitations on the total optical height are significantly relaxed.

In digital holographic microscopy (DHM), the superposition of the object and the reference waves is recorded by a CCD array and is reconstructed by numerically propagating the optical field along the direction perpendicular to the hologram plane (z direction) in accordance with the laws of diffraction. Once the complex field is calculated, its phase \( \varphi(x, y) \) can be calculated. If the light wave reflects from an object, then its surface is described by a height map \( h(x, y) \), which is determined from the phase map \( \varphi(x, y) \) of the holographic reconstruction at a given wavelength by

\[
h(x, y) = \frac{\lambda}{2\pi} \varphi(x, y).
\]

Here \( \lambda \) is equal to 1/2 of the light wavelength, as the light travels to the surface and then is reflected back. If the object is a mostly transparent cell on a reflective substrate, such that the light propagates through it, reflects from the substrate, and propagates back, then the physical height is

\[
h(x, y) = \frac{\lambda}{2\pi} \frac{\varphi(x, y)}{(n-n_0)}.
\]

where \( n-n_0 \) is the refractive index difference between the cell and the surrounding medium. The phase images of objects with the optical thickness variation greater than \( \lambda \) are ambiguous and contain \( 2\pi \) discontinuities. Such phase images need to be unwrapped. A great number of software algorithmic approaches to unwrap phase have been developed.

Previously, multiple-wavelength techniques that remove the \( 2\pi \) discontinuities have been introduced. The methods are based on the comparison of the phase maps \( \varphi(x, y) \) obtained with different wavelengths, as the discontinuities in those phase images do not occur at the same places. Suppose the object under study is a reflective surface that is being imaged by using two beams with different wavelengths and each wavelength is smaller than the overall object height. Then, the height map is

\[
h(x, y) = \frac{\lambda_1}{2\pi} \varphi_1(x, y) + \frac{\lambda_2}{2\pi} \varphi_2(x, y) + \lambda_1 m_1(x, y) + \lambda_2 m_2(x, y),
\]

where \( m_1 \) and \( m_2 \) are the unknown nonnegative integer numbers at a point \( (x, y) \). From these equations, we have

\[
\frac{\varphi_1(x, y)}{2\pi} + m_1(x, y) = \frac{h(x, y)}{\lambda_1},
\]

\[
\frac{\varphi_2(x, y)}{2\pi} + m_2(x, y) = \frac{h(x, y)}{\lambda_2}.
\]

Subtracting the second equation from the first in Eq. (4) gives

\[
h(x, y) = \frac{\lambda_2 \lambda_1}{\lambda_2 - \lambda_1} \left[ \frac{\varphi_1(x, y) - \varphi_2(x, y)}{2\pi} + m_1(x, y) - m_2(x, y) \right].
\]

The term \( \Lambda_{12} = \frac{\lambda_2 \lambda_1}{\lambda_2 - \lambda_1} \) is known as the synthetic or beat wavelength. Assuming that \( \lambda_2 \) is bigger than \( \lambda_1 \), then \( m_1 \) cannot be smaller than \( m_2 \). If we also assume that the total height of the object is less than the synthetic wavelength, then the term

\[
\frac{\varphi_1(x, y) - \varphi_2(x, y)}{2\pi} + m_1(x, y) - m_2(x, y)
\]

must be nonnegative and less than 1.
Because \(0 \leq \varphi_{1,2}(x, y) \leq 2\pi\), the first term in brackets is between \(-1\) and \(+1\). Therefore, depending on whether this term is positive or negative, the term \(m_1(x, y) - m_2(x, y)\) must be equal to either 0 or 1.

Thus, subtracting the two phase maps obtained from the same object using two wavelengths of the input light beams and adding \(2\pi\) whenever the phase difference is negative yields a new phase map, which corresponds to the beat wavelength \(\Lambda_{12}\). The height image of the object can then be obtained. In practice, however, this method, while extending the range of unambiguous phase measurement, also increases error [6]. For example, if the laser wavelengths of 633 and 532 nm are used to generated two phase maps, we then have \(\Lambda_{12} = 3.3 \mu m\). If we assume that each phase measurement has uncertainty of 0.1 rad, an optical thickness profile map obtained from a single wavelength will have an error of only about 10 nm. However, the error of the extended range optical thickness profile will be approximately 70 nm, which is unacceptable high. This severely limits the applicability of this method for any measurements obtained by the dual-wavelength method. It is possible to use the dual-wavelength profile as a rough guide to correct one of the original single-wavelength maps. However, this method fails if the noise in the phase map, obtained by each of the single wavelengths, exceeds a certain limit [6]. It also requires the two wavelengths to be sufficiently far apart, reducing the total measurable range because of the decrease of \(\Lambda_{12}\).

Here we present a method for phase unwrapping that bypasses the generation of the synthetic wavelength profile. This method also allows for increase of the measurable height range. Rearranging Eq. (4), we have

\[
m_1(x, y) = \frac{\lambda_2}{\lambda_1} m_2(x, y) + \frac{1}{2\pi} \left( \frac{\lambda_2}{\lambda_1} \varphi_2(x, y) - \varphi_1(x, y) \right).
\]

Theoretically, in the absence of any uncertainty in phase and wavelength measurements, this linear equation of \(m_1\) in terms of \(m_2\) can be solved exactly for integer values \(m_1\) and \(m_2\). Moreover, unless the two wavelengths have a common divisor (e.g., the wavelengths are exactly 500 and 600 nm), the range for unambiguous height measurements extends to infinity, as there is only one set of integers, \(m_1\) and \(m_2\), where Eq. (1) holds exactly true. When the real phase measurements are involved, this linear equation of \(m_1\) in terms of \(m_2\) can be used to determine the most likely integers \(m_1\) and \(m_2\) by rounding

\[
m_1(x, y) = \text{round} \left( \frac{\lambda_2}{\lambda_1} m_2(x, y) + \frac{1}{2\pi} \left( \frac{\lambda_2}{\lambda_1} \varphi_2(x, y) - \varphi_1(x, y) \right) \right).
\]

where \(m_2\) is such that the difference between Eqs. (5) and (6) is at the minimum [i.e., the left side of Eq. (6) is closest to an integer]. Equation (6) is a special case of Eq. (5), where the difference between \(m_1\) and \(m_2\) is allowed to be either 0 or 1, i.e., \(m_1 = \text{int} \left[ \frac{\lambda_2}{\lambda_1} m_2 \right]\). However, in a general case, the object’s overall height is different from \(\Lambda_{12}\), and there is no need to set this limit for the value of \(m_2\). The range of allowed \(m_2\) values can be selected based on the overall height of the sample, which can be estimated by observing the wrapped phase image and counting the number of observable phase jumps.

Because the wavelengths of the input laser light beams can be measured very accurately, the slope of the line \(m_1(m_2)\) (Fig. 1) has much smaller uncertainty than the y intercept, which is dependent on the measured phases for each point of the image. By finding the value of \(m_2\) at which \(m_1\) is closest to the nearest integer, we can determine the correct number of integer wavelengths and recreate the optical thickness profile of the original object.

In practice, there may be areas in one or both of the single-wavelength images that are corrupted by excessive noise, such that the algorithm will not pick the right \(m_2\) but may select the neighboring value instead. The likelihood of this increases if the two wavelengths are closer to each other, as then the slope in Fig. 1 is closer to 1, and different values of \(m_2\) are more difficult to distinguish. Therefore, once the phase unwrapping is complete, if some of the areas in the image are noisy, the pixels in these areas need to be shifted back to their proper places by using a software algorithm that looks for \(\lambda_2\) jumps in the final image and corrects them by shifting the pixels up or down.

Figure 2 shows the phase image of two KB cells, obtained by our dual-wavelength digital holographic microscope. The light from two pigtail laser diode systems (675
and 635 nm) is combined using a beamsplitter and is then split again into object and reference arms. The two beams are recombined, and the interference pattern is imaged on a CCD array using a water immersion objective. The curvature mismatch between the reference and object waves is compensated numerically \[7\].

It is clear from the single-wavelength images [Figs. 2(a) and 2(b)] that the object is about two wavelengths high, and, therefore, there is no need to extend the range beyond that. Figures 2(c) and 2(d), obtained by our linear regression dual-wavelength method, present the final unwrapped image, free of discontinuities.

If the object is many wavelengths high, this method is still capable of unwrapping the phase and producing the final height map free of discontinuities. Figure 3 shows two SKOV-3 ovarian cancer cells on a substrate, which was tilted with respect to the optical axes in order to increase its overall height range to demonstrate this method. While the object in Fig. 3 is twice the height of the synthetic wavelength \(\Lambda_{12}\), it is still unwrapped correctly, which would have been impossible previously using only two wavelengths \[4\]. The height range at which the unambiguous phase imaging can be performed is still somewhat limited by noise, because if the object is very thick (the maximum \(m_2\) is allowed to be large, and so the graph in Fig. 1 includes many minima), there is more room for error; in that case, determining the right value of \(m_2\) can be problematic. However, a flexible trade-off is achieved between the higher height range and higher accuracy.

In conclusion, our dual-wavelength unwrapping is well equipped to deal with complicated phase topologies, and it is computationally fast (only limited by the speed at which the angular spectra for both wavelengths are calculated). The method’s application is not limited to DHM, but it also can be applied to other phase-imaging techniques, such as phase-shifting interferometry. By extending unambiguous optical phase-imaging methods to objects of different heights, the linear regression dual-wavelength unwrapping method makes phase imaging much more practical, allowing 3D measurements of a wide variety of biological systems and microstructures.

**References**