Quantitative DIC microscopy using a geometric phase shifter

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ABSTRACT

In this paper we investigate the use of a geometric phase-shifting (GPS) technique which allows us to convert conventional transmission or reflection differential interference contrast (DIC) microscopy into a quantitative mode. A phase-shifting algorithm is employed to extract the specimen phase gradient from the mixture of phase and amplitude information which is common in DIC. Fourier techniques are then used to recover the exact phase (i.e. optical path length variations) throughout the biological specimen viewed. In addition to this quantitative "phase map," we demonstrate that the GPS process simultaneously yields an "amplitude-only" representation in which various absorption and transmission properties of the specimen are displayed as intensity variations in the image, similar to brightfield microscopy. These two resulting images can then be analysed or further processed in a number of ways that are not possible with conventional DIC and which improve the microscopist's ability to correctly identify, interpret and measure features in the specimen.

Key words: 3D microscopy, interference, DIC, phase-shifting, image processing, biomedical microscopy

1. INTRODUCTION

In the biological and medical fields, there exists a need for imaging of transparent or semi-transparent specimens. Advances in optical microscopy have evolved which allow variations in the phase of the illuminating light caused by inhomogeneities in the specimen to be transformed into intensity variations in the image. Differential interference contrast (DIC) is today generally considered the best available phase imaging method and is in widespread use, having surpassed Zernike phase contrast in popularity. However, due to inherent nonlinearities (see following section), it can only be used for qualitative imaging and is hence not suited for measurement.

DIC was patented in 1953 by Georges Nomarski and is based on principles of optical interferometry.[1] In the DIC microscope, prior to the specimen, a plane polarised illuminating beam is split by a Wollaston prism into two orthogonal component polarisations with a slight lateral shear (lateral displacement) between them (Fig. 1a). The two components both illuminate the specimen and both respond to specimen features which alter the optical path (Fig. 1b). For example, higher refractive index regions or those of greater thickness cause the light beam leaving the specimen to have its wavefront impregnated with the object phase information in the form of phase lags (which modulate the wavefront). After the specimen, the lateral shear is removed by a second Wollaston prism and the two components are made to interfere by an appropriately oriented polariser. The difference in optical path length between the two beams for the various specimen features is then revealed as an intensity variation, and produces a visual pseudo-3D (*bas relief*) effect.

DIC has several distinct advantages over other imaging modes of microscopy such as brightfield or phase contrast. Besides being able to image phase gradients, it also correctly responds to specimen amplitude variations so that specimens with some absorbing regions (e.g. pigmented or stained areas) can be visualised as well as transparent regions. DIC also can be utilised with very high numerical aperture (NA) optics and has the benefit that its highest spatial frequencies tend to be largely confined to the plane of focus of the microscope. This produces a "sectioning" effect which is useful for rejecting some of the blurring contributions from the out-of-focus planes. However, this should not be confused with the "optical sectioning" response of confocal reflection DIC which sections equally well for all spatial frequencies.[2]

In conventional DIC microscopy, the linearity of response, the contrast, the overall brightness, the phase/amplitude information ratio and other attributes of the final image are dependent on the bias phase which is controlled by the lateral position of the objective Wollaston prism. For the best determination of the phase of the specimen, a "phase only" image is obtained from DIC for a bias phase of 0; but at this setting, the response is highly non-linear and the image exhibits bright regions wherever positive *or negative* phase gradients occur. This phenomenon is known as fringe-doubling and causes confusion in interpretation of phase gradients from the output DIC image.

The most linear response to specimen phase gradients occurs at a bias phase setting of 45 degrees. However, the contrast created by a phase gradient is lowest at this setting, and there is a combination of phase and amplitude information present

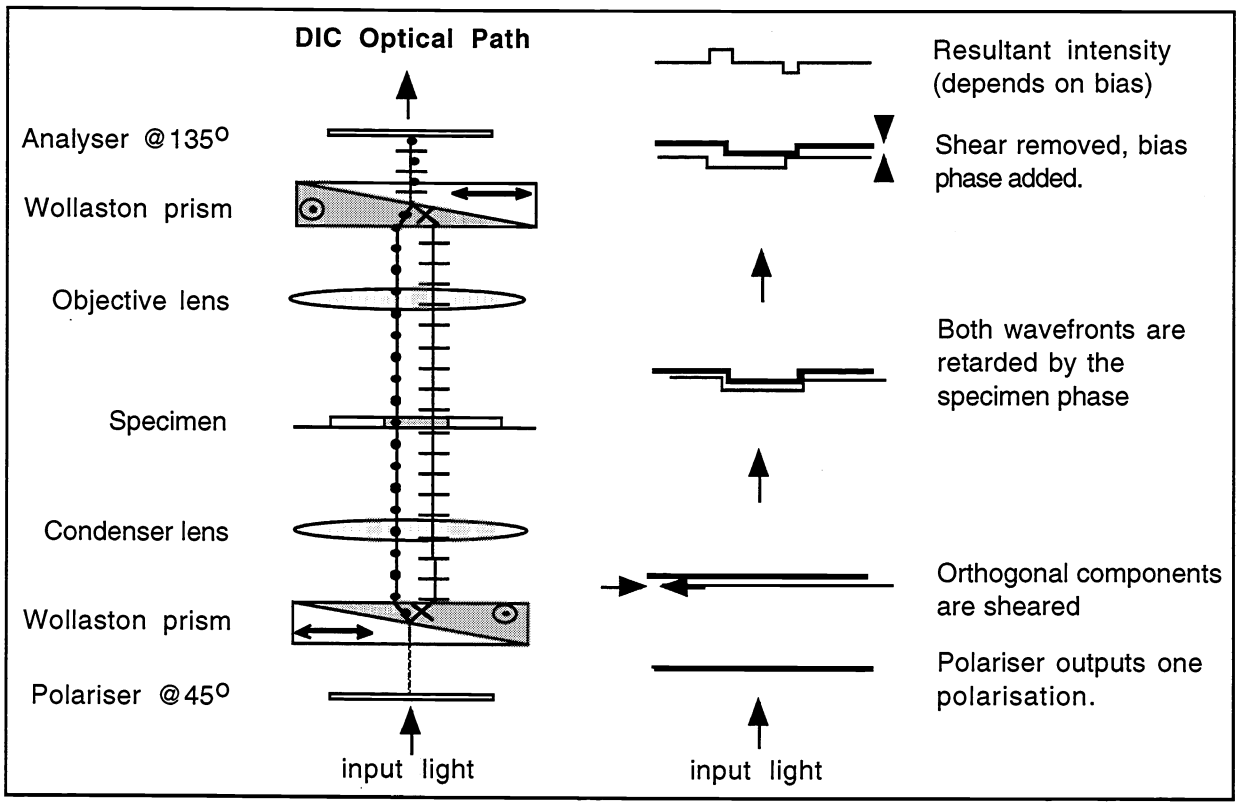

Figure 1. Schematic diagram of the optical path through a transmission DIC microscope showing the optical components (left) and their effects on the orthogonally-polarised wavefronts (right) which ultimately interfere to form the *bas relief* image.[3]

in the final image which makes it difficult to measure phase alone. By comparison, phase-shifting methods are well known for their ability to isolate phase information and measure it accurately. It is this fact that led us to the implementation of our phase-shifting technique for DIC microscopy.

2. THEORY

2.1 Differential interference contrast

In an earlier paper[2] we develop a theoretical model for the imaging of two types of objects (weak objects or pure-constant-phase-gradient objects) in a conventional DIC microscope. In this model, we assume a partially-coherent optical system which is correct for our microscope where the condenser and objective numerical apertures are equal (i.e. the pupils of both lenses are equally filled with light). Although the model covers only two types of objects, it is sufficient to demonstrate the effect of specimen phase gradients on the optical system and hence on the corresponding phase gradient transfer function. Specifically, these types of phase-gradient objects cause the cone of light emanating from the specimen in the detection (imaging) path to be tilted. This causes a lateral shift of the beam at the lens pupil which is then no longer totally filled with light. This so-called "vignetting" effect is the basis for our model in which the phase gradient transfer function can be calculated as the area of overlap of the two circles from the illumination and detection pupils of the optical system (Fig. 2).

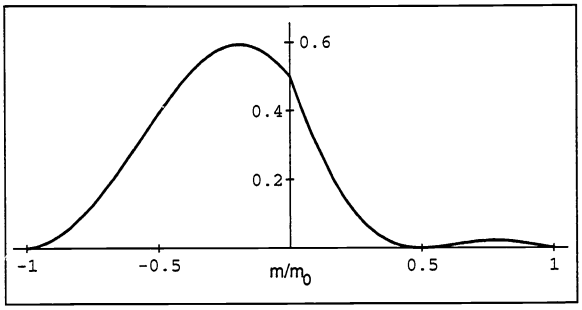

Figure 2. The phase gradient transfer function for a DIC system with equal condenser and objective apertures and a bias phase of 45 degrees, showing signal strength as a function of normalised phase gradient m/m_0. Vignetting causes nonlinearity.[2]

By considering this vignetting effect, we show that the phase gradient transfer function and hence the conventional DIC optical system no longer gives a linear response for all object phase gradients (i.e. for all spatial frequencies). This non-linear response is what prevents *quantitative* measurements of specimen phase and the directions of phase slopes from being obtained from conventional DIC images. In order to remedy this difficulty, we must turn to phase-shifting techniques.

2.2 Phase shifting in DIC

In a DIC microscope, for a specimen where the amplitude, a, and phase, θ, varies across the specimen, the complex amplitude, t, of the two orthogonally polarised beams may be written:

$$\begin{aligned} t_1 &= a_1 e^{i(\theta_1 - \phi)} \\ t_2 &= a_2 e^{i(\theta_2 + \phi)} \end{aligned} \tag{1}$$

where 2ϕ is the prism-induced phase difference (bias) between the two beams, and θ_1- θ_2 is the phase difference caused by the specimen phase gradient.

The intensity of the final image will be given by the modulus squared of the addition of the complex amplitudes:

$$I = |t_1 + t_2|^2 = (t_1 + t_2)(t_1^* + t_2^*) \tag{2}$$

$$= a_1^2 + a_2^2 + 2a_1a_2\cos(\theta_1 - \theta_2 + 2\phi) \tag{3}$$

and if we let the object induced phase difference (which is the variable of most interest to this work) be represented by θ_1- θ_2 = $\Delta\theta$, then the above equation contains four variables: a_1, a_2, $\Delta\theta$, and 2ϕ. For DIC, we may set 2ϕ by changing the bias phase using a Wollaston prism or we may vary the introduced phase by some form of phase shifting. Mathematically, it is not relevant *how* the phase is shifted, only the amount by which it is shifted.

From a single image of a specimen, we can only obtain measurements of intensity, which are composed of a mix of specimen amplitude and phase information and also depend on the phase offset. In our GPS-DIC system, we acquire four images, in each of which 2ϕ is incremented by 90 degrees.[4] Consequently we obtain four linearly independent equations* from which can be found the phase gradient in the specimen:

$$\begin{aligned} I_0 &= a_1^2 + a_2^2 + 2a_1a_2\cos(\Delta\theta) \\ I_{90} &= a_1^2 + a_2^2 + 2a_1a_2\sin(\Delta\theta) \\ I_{180} &= a_1^2 + a_2^2 - 2a_1a_2\cos(\Delta\theta) \\ I_{270} &= a_1^2 + a_2^2 - 2a_1a_2\sin(\Delta\theta) \end{aligned} \tag{4}$$

solving for $\Delta\theta$:

$$\begin{aligned} I_{90} - I_{270} &= 4a_1a_2\sin(\Delta\theta) \\ I_0 - I_{180} &= 4a_1a_2\cos(\Delta\theta) \end{aligned} \tag{5}$$

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* The reader may notice that only three independent measurements are required to solve for all three unknowns, so that the phase could be measured with only three images. The reason for using four in this initial research is that the four-step algorithm is quite simple to analyse and the phase-stepped images cover a greater range of phase bias for investigation of the mode. For later implementation, a similar three-step algorithm would likely be sufficient.

so that:

$$\Delta\theta = \tan^{-1}\frac{I_{90} - I_{270}}{I_0 - I_{180}} \tag{6}$$

which is the specimen phase difference (phase gradient) between the two beams. A similar derivation yields an equation for specimen amplitude information. It is apparent from the above that the phase shifting algorithm is exact. The accuracy of the phase measurements will be dependent only on the accuracy of the phase shifting optics and recording apparatus.

If measurements of optical path difference (Δop) or the related refractive index or thickness of regions within the specimen are required, the optical path difference can be determined from the following relationship to the phase gradient:

$$\Delta\theta = \frac{2\pi}{\bar{\lambda}}\Delta op \tag{7}$$

where $\bar{\lambda}$ is the mean wavelength of the illuminating light. It should now be apparent in theory how GPS-DIC can provide more comprehensive and accurate measurements than conventional DIC by its ability to isolate the required information from the image. However, we have not considered the means by which the phase-shift between the two beams is introduced. To do so requires some discussion of geometric phase models.

2.3 Geometric phase shifting

Geometric phase manifests itself in optics when the state of polarisation of a light beam is forced to change in such a way that it traces out a closed cycle when viewed in the space representing polarisation (which mathematically is a sphere).[5] By changing the intermediate polarisation states, it is possible to alter the polarisation cycle. A geometric phase shift is a phase shift which occurs between two polarisation states when they are cycled (translated) in different ways, even if they return to the same initial state. The geometric phase is an added phase shift, dependent only on the path taken.

In our GPS-DIC microscope configuration, we use a quarter-wave plate (QWP) to convert the orthogonal polarisations (i.e. the DIC beams) to oppositely-handed circular polarisations, once they have passed through the specimen and second Wollaston prism. Both circular components then pass through a rotatable polarising analyser, where we can precisely alter the relative paths taken by each beam's polarisation state and hence control the amount of added geometric phase shift.

3. EXPERIMENTAL SETUP

A Zeiss Axioplan microscope with DIC optics was adapted for geometric phase shifting simply by inserting a quarter-wave plate into a space above the second Wollaston prism, just below the analyser. The axis of the QWP was precisely aligned to ensure the two orthogonally-polarised imaging beams became circularly polarised in opposite directions. Phase shifting could then be obtained by way of the rotatable analyser which has an accurate scale for recording polarisation angles. This configuration is called a "Senarmont compensator" and its end result is the introduction of a phase shift between the two beam components equal to twice the angle between the analyser and the optical axis of the QWP.[6]

A GPS-DIC image was obtained by first acquiring four images, where for each successive image the analyser was advanced (rotated) by 45° (which causes a 90° phase shift). Images were frame-grabbed using a cooled CCD camera (Photometrics Nu200) which has a large dynamic range and a linear response in the visible light region. Image acquisition was followed by the application of the phase-shifting algorithm, producing a final image in which intensities represent phase *gradients* in the specimen.

4. RESULTS

Initial tests were performed using a specimen comprised of a droplet of water and an edge of a droplet of oil. Even without knowing in detail the gradients of the droplets, some assumptions about the surface could be made. The surface of a water droplet resting on the glass can be approximated by a parabola (due to the effects of gravity and surface tension). The GPS-DIC microscope setup denotes phase gradients as intensities in the image, and since the derivative of a parabola is linear, the microscope response to the droplet phase gradient should be linear across the droplet. Figure 3a shows an image of this specimen using zero-bias conventional DIC followed by a plot of intensities through a section of this image (Fig. 3b). For comparison, similar plots (Fig. 3c-d) are included for the same specimen when a 45° bias DIC and a GPS-DIC system were

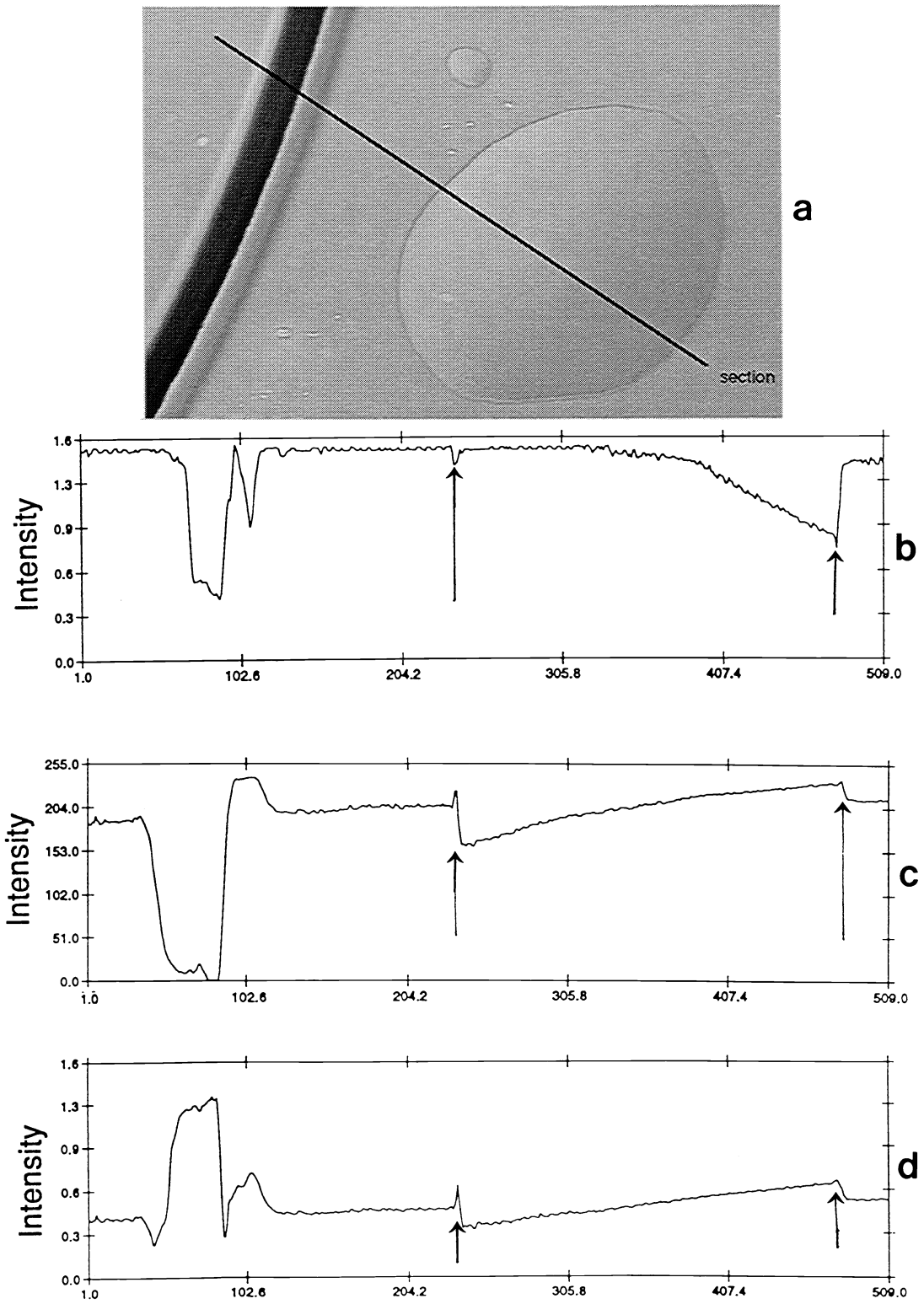

Figure 3. Linearity of response of DIC vs. GPS-DIC to a phase gradient. (a) A water droplet as a phase object specimen. (b) a plot of the intensity through the section indicated when conventional DIC optics are used with a zero bias setting. Note the extreme non-linearity to the droplet region (between arrows). (c) same as (b) but with 45^{o} bias which shows better linearity. (d) a plot from a GPS-DIC image shows the most linear response to the phase gradient.

used to form the image. The bias settings (which we recall are the introduced phase offsets between the two interfering beams) for the conventional DIC measurements were chosen because they provide the best approximations to an image containing only phase information (0 bias phase) or one at the most linear setting (45 degree bias phase). It can be seen that the response in the 0 bias phase image (Fig. 3b) is largely non-linear, the plot does not represent the actual phase gradients in the specimen at all well. The response for the 45^{o} bias phase DIC image (Fig. 3c) is better in terms of a linear intensity response, however, while it is not apparent upon observation, DIC theory tells us that this image is composed half of phase information and half of amplitude. By comparison the GPS-DIC image plot (Fig. 3d) gives a more linear response across the droplet than the most linear obtainable image with conventional DIC. In addition, the phase-shifting technique provides only the phase gradient information instead of a complicated mixture of phase and amplitude, and it would still provide good phase measurements even if the droplet had varying transparency.

To begin investigating the potential of GPS-DIC for imaging unstained biological preparations, we conducted preliminary experiments using immature orchid embryos (*Microtis parviflora*) fixed and mounted in lacto-phenol which also acts as a clearing agent, making the specimen more transparent. Initially, two datasets were acquired at two different rotations of the specimen in order to compensate for the problem that DIC only gives phase gradient information in the direction of its prism lateral shear. Each dataset was comprised of five images, four at different rotations of the GPS-DIC analyser and one control image which had no QWP but instead was optimised for conventional DIC. The four GPS-DIC images were processed using an algorithm described by Eq. 6 which produces a resulting image where intensity is linearly proportional to the object phase *gradients* along the direction of lateral shear. In addition, a second algorithm was employed to extract the specimen amplitude from the four GPS-DIC images in each dataset. Figure 4 shows the resulting images from one angle of rotation of the specimen. Figure 4a is the conventional DIC image which contains a mix of phase and amplitude information, including, for example, a large absorbing "dirt" spot in the upper left background region. Figure 4b shows the result of the GPS-DIC phase gradient calculation, which is similar in appearance to 4a, however certain differences can be noted, including the removal of the amplitude components such as the dirt spot at upper left. Figure 4c is the amplitude-only image from the GPS-DIC process which now clearly shows the dirt spots in the optical path and it also shows the variation in illumination intensity across the field of view.

In order to obtain the "true" phase of the specimen from the GPS-DIC datasets, we have explored a number of techniques for further processing the images. Our initial approach was to integrate the image in the direction of shear. Numerical integration has its drawbacks however, most notably, the loss of high frequency information in the gradient image and also a streaking effect due to the unknown constant of integration.

We found that a more effective approach for visualising the specimen phase was to use a Hilbert transform which is similar to an integration, but does not attenuate high spatial frequencies.[7] This fact, however, means that the Hilbert transform does not allow us to measure specimen phase exactly since it maintains the high frequency boost characteristics of a DIC optical system, unlike an integration which acts like a low-pass filter. Although the Hilbert transform is not strictly the best means to obtain the quantitative phase of the specimen, it does show the correct sign for phase gradients (e.g. upward or downward slopes) and hence it is more effective in providing information close to the true phase than is possible by using conventional DIC alone.

One advantage of processing the images to obtain the actual object phase is that we can then explore ways to visualise the results. One method is shown in Fig. 5 which is a pseudo-3D view of the Hilbert-transformed versions of Figures 4a and 4b. These were created using a surface plot function (NIH Image) in which image greyscales are plotted as surface height. To aid visualisation, the contrast was enhanced after the surface plot was applied so as not to alter the phase information. The two images are similar but do show some significant differences, despite their overall spikey appearance due to the high spatial frequency boost of the optical system. For example, the conventional DIC image has several anomalous tall white spikes along the cell walls which are less prominent in the GPS-DIC image. In addition, the phase-shifting DIC image shows the central multi-celled embryo region as being thicker and more rounded which is probably more accurate based on our knowledge of the specimen preparation. Although these preliminary examples are very complex and difficult to interpret further, they do illustrate the potential for the GPS-DIC technique to accurately portray specimen features, especially if some knowledge of the specimen thickness or refractive index is available.

5. DISCUSSION

The results shown in Figure 3 were the first results to come out of this project which support the initial proposal that the GPS-DIC system can indeed linearise the phase response of the conventional DIC microscope and isolate the information of importance. A full statement of success could not, however, be made at this point due to the fact that the actual phase gradients in the droplet were not known, although the approximation to a parabolic surface for the droplet was reasonable. To remedy this imprecision, we conducted further tests using reflection GPS-DIC optics and a specimen comprised of sections

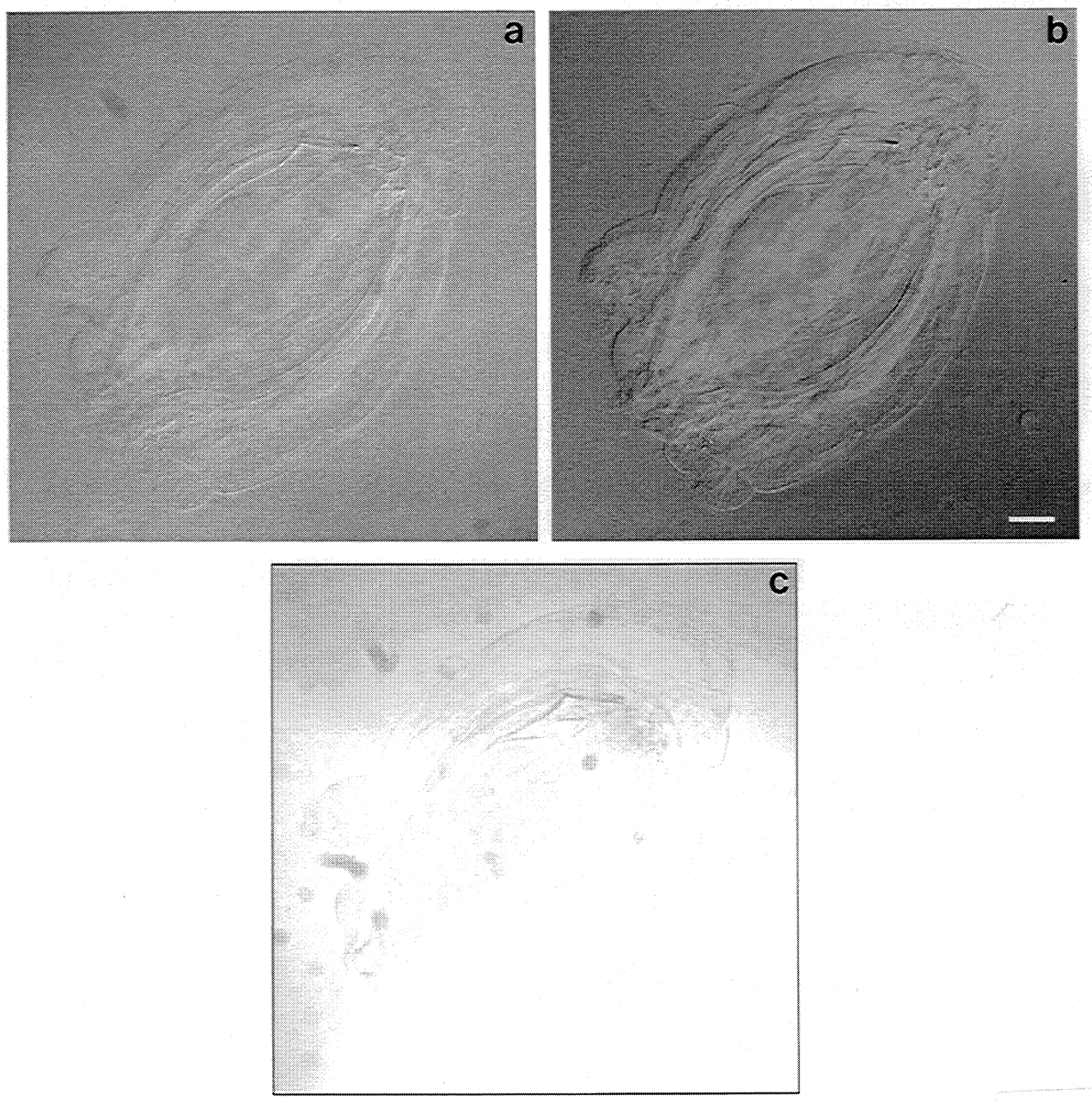

Figure 4. Comparison between conventional and GPS-DIC images of an immature orchid embryo (*Microtis parviflora*). (a) Conventional DIC, (b) the phase gradient image recovered by GPS-DIC and (c) the specimen amplitude information (again by GPS-DIC). Note the separation of amplitude information such as the dirt spots in (c) from the phase information in (b) using the GPS-DIC technique. Scale=20 μm.

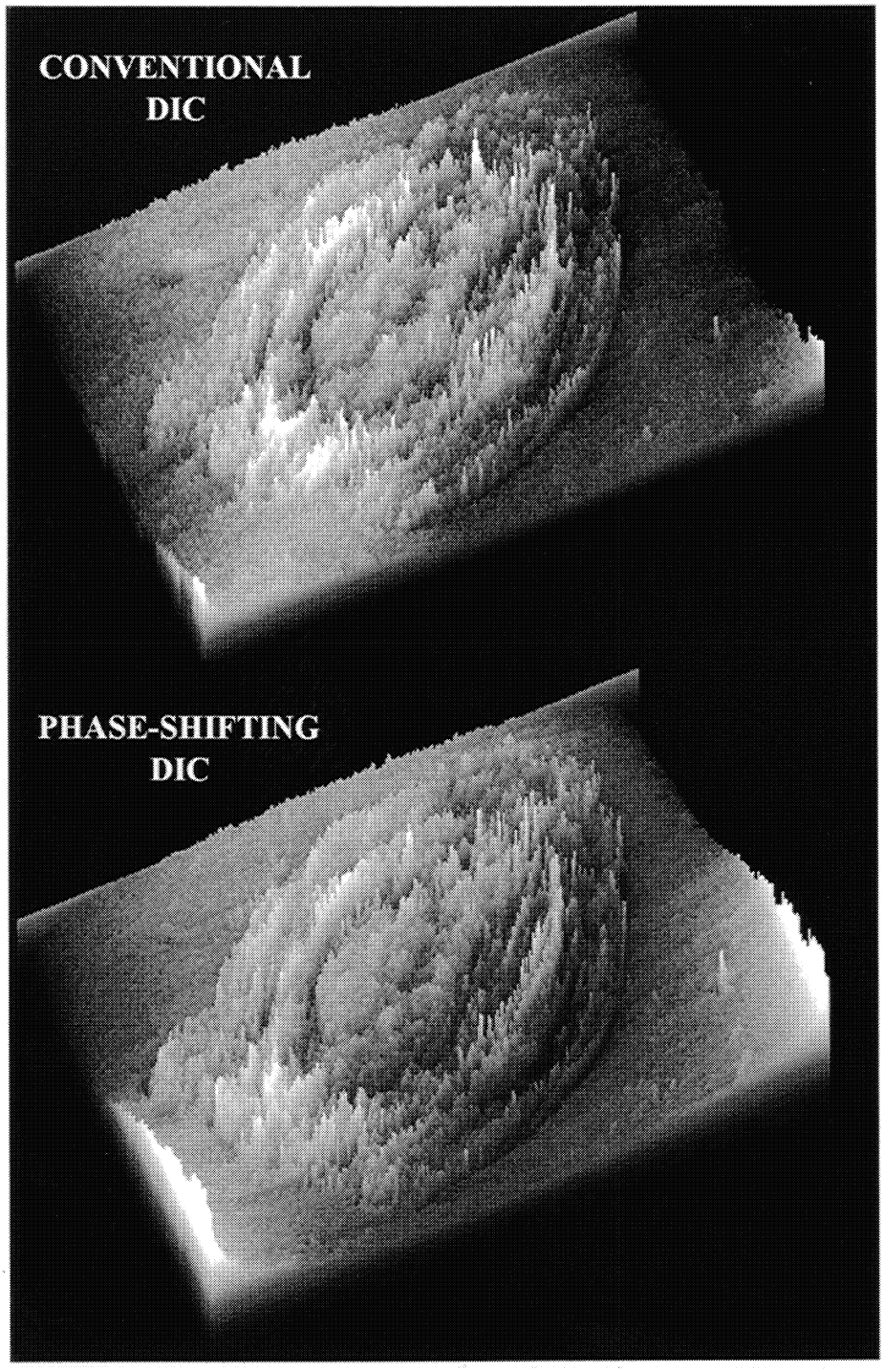

Figure 5. Pseudo-3D projections of Figures 4a and 4b. The phase-shifting DIC image shows a more uniform thickness and fewer anomalous spikes than the conventional DIC. Prior knowledge of the orchid embryo specimen indicates it should have similar refractive index throughout except possibly for the cell walls; hence the GPS-DIC image appears to give a more accurate thickness representation.

of a gold-coated Fresnel lens whose surface slopes could be accurately calculated. The resulting GPS images from this sample showed the predicted linear response for the specimen slopes (for angles up to 45^{O} to the specimen plane). By comparison, conventional DIC images gave non-linear responses for the gradient measurements.

To summarise, the following is a list of some of the specific advantages of the GPS-DIC technique:

- A precise representation of either the phase *gradients* or the true phase of the various features in the specimen can be obtained
- If some *a priori* knowledge of specimen features is available, the phase image can be further analysed to give a measure of the specimen thickness (if it is known to have the same refractive index throughout) or conversely a measure of the refractive index of various features (if their relative thickness is known from say a confocal reflection series).
- Ambiguities common in conventional DIC images such as the direction of slope of feature boundaries (i.e. whether a surface slopes upwards or downwards) are removed. Non-linearities in response to widely-varying phase gradients are also removed.
- Imperfections such as uneven illumination or dirt spots in the optical system or on the CCD camera do not appear in the phase image provided the dirt is only attenuating the beam and not also acting as a phase object.
- A precise representation of the specimen absorption can be obtained at the same time as its phase
- 3D visualisation techniques (such as isometric projections) can be employed in some instances to represent specimen thickness.
- In principle, GPS-DIC is achromatic and can be used in white light providing appropriate waveplates are employed.

Some problems also exist with the GPS-DIC imaging technique and these are outlined below with some suggested solutions:

- The image phase gradient is given across the specimen only in the direction of the DIC prism lateral shear. This drawback can be overcome by rotating the specimen on the microscope stage and taking another set of GPS-DIC images.
- Conventional DIC images are not true "optical sections" in the sense that confocal images are. Therefore the resulting thickness representations are limited in that they do not contain full 3D "voxels." Investigations into acquiring GPS-DIC images from multiple planes of focus through the specimen followed by image processing and feature extraction procedures are being explored.
- Currently the GPS-DIC process of acquiring four images at four positions of the polarising analyser is somewhat slow. Theory predicts that only three images are required and techniques are being explored to automate the image acquisition process. This should make imaging of living specimens feasible.
- The Hilbert transform used in the initial experiments gives a somewhat imprecise representation of the specimen phase due to its maintaining the high spatial frequencies of the DIC optical system. Further investigations of integration (and differentiation) techniques are underway so that we can recover quantitative phase and hence measure specimen thickness or refractive index.

Future investigations involve incorporating the GPS-DIC system into our experimental "multiple-optical-mode" confocal microscope. Experiments in confocal GPS-DIC in both transmission and reflection will further explore the feasibility of using this technique for precise measurement of specimen feature refractive index throughout the entire specimen depth.

6. CONCLUSION

We have presented a new method for obtaining precise phase measurements from a wide variety of specimens using a geometric phase shifting technique in conjunction with a conventional DIC microscope. This technique is based on the principles of a Senarmont compensator and is extremely simple to use, requiring only the rotation of a polarising analyser while acquiring three digital images. Phase gradient as well as specimen amplitude (absorption) information can be obtained following straightforward image calculations. With some further processing in Fourier space the true phase of the specimen can be obtained. This makes possible the visualisation and measurement of phase specimens such as living cells with minimum disruption to cellular mechanisms. In addition, with some prior knowledge (such as specimen thickness obtained with a confocal reflection microscope) the refractive index of specimen features can be obtained.

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8. REFERENCES

1. Pluta, M., *Advanced Light Microscopy, vol 2: Specialised Methods*, Elsevier, Amsterdam, pp. 146-197, 1989.

2. Cogswell, C. J. and Sheppard, C. J. R., "Confocal differential interference contrast (DIC) microscopy: including a theoretical analysis of conventional and confocal DIC imaging," *J. Microsc.* **165**, 81-101, 1992.

3. Oldfield, R., *Light Microscopy, An Illustrated Guide*, Wolfe, London, 1994.

4. Creath, K., *Phase Shifting Holographic Interferometry*, Springer-Verlag, pp. 109-150, 1994.

5. Berry, M., "Anticipations of the Geometric Phase," *Physics Today*, pp. 34-40, December 1990.

6. Hariharan, P., "The Senarmont Compensator: an early Application of Geometric Phase," *J Modern Optics*, **40**, 2061-2064, 1993.

7. Cogswell, C. J., Larkin, K. L., Arnison, M. R. and O'Byrne, J. W., "3D Fourier analysis methods for digital processing and 3D visualization of confocal transmission images," *Proceedings SPIE*, **2412**, 230-235, 1995.