Optical Phase Microscopy: Quantitative Imaging and Conventional Phase Analogs

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BIOGRAPHY

Keith A. Nugent is Professor and Head of the School of Physics, University of Melbourne, Australia. Prof. Nugent was recently elected to the Australian Academy of Science, is one of only 15 Australian Federation Fellows and has received numerous awards for his work. His research interests are in X-ray and visible optics, and he is a key inventor of the Quantitative Phase Imaging technology.

ABSTRACT

Experienced microscopists are familiar with the process of slightly defocusing the microscope to visualize (edge) structure within transparent samples. This is a method of phase contrast imaging. We present a method of quantitative optical phase microscopy that quantifies this phase contrast effect. Starting with conventional, digital, bright field images of the sample, our non-recursive algorithm provides quantitative phase information that is completely decoupled from the bright field intensity image. This independent phase and intensity information is then used to emulate a range of conventional experimental phase visualization modalities.

KEYWORDS

quantitative phase imaging, phase contrast, DIC, Hoffman modulation contrast, dark field, transport of intensity equation

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INTRODUCTION

A sample under a microscope affects the transmitted light by absorption and/or scattering (changing amplitude) and refraction (changing phase). The intensity and phase information constitute a signature of the amplitude and phase structure of the sample through which the radiation has passed. Transmission microscopy techniques have been developed that utilize either, but not both, of these effects to improve on sample contrast. For example, bright field microscopy is employed for samples that have sufficient amplitude contrast, either natural or induced by staining. Alternately, dark field techniques image the scattering from samples. In the case of transparent samples, amplitude contrast is of limited value, and the only useful structural information is available in the induced phase alterations. Therefore, techniques have been developed to provide contrast enhancement and better visualization of transparent samples displaying sufficient phase contrast. These phase visualization techniques include, Zernike phase contrast (conventionally referred to as standard 'phase'), Normarski differential interference contrast (DIC), and Hoffman modulation contrast (HMC).

QUANTITATIVE PHASE IMAGING - BASIC PRINCIPLES

The quantitative phase imaging technique is the result of research performed at the University of Melbourne [1] utilizing the Transport of Intensity Equation (TIE) [2]. The TIE describes the inter-relationship between intensity and phase. Fig1 displays an example of this inter-relationship, showing how the sample modifies the wavefront leaving the sample and illustrating the effects of this (phase) modification on the intensity distribution seen at planes further downstream. In effect the TIE says that knowledge of the intensity and phase in a plane defines the intensity derivative. The intensity derivative is then used to calculate the intensity (and phase) in any subsequent plane.

Conversely, if, by experiment, the intensity is measured over a series of planes (two or more), the intensity derivative may be calculated. In this situation, the inverse problem can be solved to determine the phase in the central plane of the series. This quantitative phase analytical technique has been successfully applied to optical [3,4] and electron microscopy [5], and X-ray [6,7] and neutron [8] imaging.

Conventionally, the quantitative measurement of the phase of microscope samples involves the experimentally demanding interference microscopy technique using exacting optical components. However, more importantly, the necessity for coherent light precludes the use of non-coherent light with it's attendent improvements in spatial coherence. On the other hand, quantitative phase imaging is a non-interferometric solution and therefore has more relaxed experimental conditions than interferometric techniques. Indeed, precise phase measurement is possible for highly structured samples using non-uniform, polychromatic, partially coherent radiation.

QUANTITATIVE PHASE IMAGING - PRACTICAL IMPLEMENTATION

The technique of quantitative phase imaging entails taking a series of three, through-focal, bright field images, consisting of an in-focus bright field image, and a pair of slightly (still within the depth of field) defocused images either side of this. The in-focus position is defined by the image of sharpest detail. It is not necessarily the plane of best contrast, and

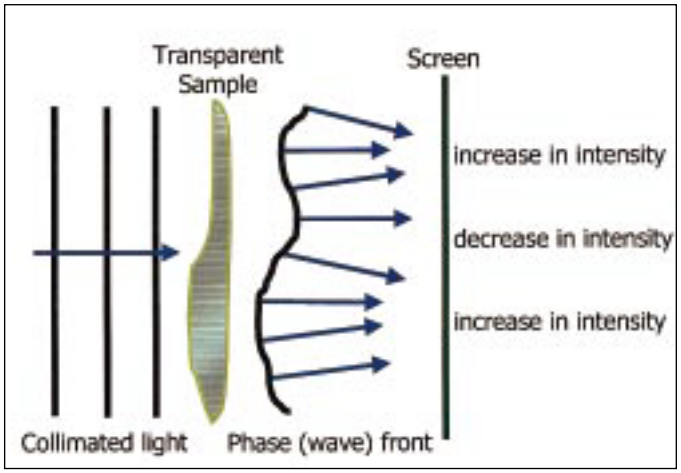

Figure 1: Schematic of propagation-based phase contrast imaging, showing the effect of the specimen refractive structure on the transmitted wave (phase) front and the resultant change in intensity after propagation.

is commonly the plane of least contrast in brightfield imaging. The amplitude contrast of the sample is defined by the central (in-focus) bright field image. Using the quantitative phase imaging technique, we can then solve for the phase across the central in-focus plane. This corresponds to the phase (optical thickness) of the sample within the depth of field of the optics. The image displays the same spatial resolution as a conventional brightfield image.

A PRODUCT FOR QUANTITATIVE PHASE IMAGING

Iatia has developed a self-contained product [9], to implement quantitative phase imaging for optical microscopy. The software captures conventional bright field images and produces independent intensity and phase information. Once the image series has been captured, all other processing is performed in the software environment. Moreover, our software uses the intensity and phase information to generate analogs to conventional phase visualization techniques, and to generate new imaging visualizations. It also incorporates a suite of image analysis and manipulation tools. Essentially, the software automates the acquisition of the brightfield image series, calculates the phase image and can then generate a wide range of analog images including DIC, standard phase, darkfield and HMC.

QUANTITATIVE PHASE IMAGING OF A CELLULAR SPECIMEN

In the investigation of viable cellular specimens the value of phase analysis and visualization becomes particularly apparent. Our studies of the large filamentous freshwater alga, *Spirogyra,* demonstrate the utility of this technology.

Spirogyra comprises individual cells joined to form an unbranched cylindrical filament with one or two spirally wound chloroplasts. Most of the interior of the cells is occupied by a large fairly transparent vacuole, in which the central nucleus is suspended by cytoplasmic strands [10]. These filaments consist of both light absorbing and phase altering components which can be differentiated using quantitative phase imaging. For this study, we investigated freshly harvested unstained *Spirogyra*, mounted in aqueous solution on polylysine-coated glass supports.

Filaments were initially visualized under standard bright field (Koehler) conditions on a Zeiss Axioscope 2 MOT or Axiovert 100 M light microscope. Digital images were captured using two different 12 bit black and white CCD camera types (1600 x 1200 pixel Diagnostic Instruments SPOT-RT and 1300 x 1030 pixel Roper Photometrics CoolSnap FX). The QPm software was used to acquire defocused image sets at different magnifications (see the objective information in Figure captions) from which the phase images could be recovered computationally. Images were also acquired using Normaski DIC, standard phase and darkfield optical configurations for comparison with the QPm generated image analogs.

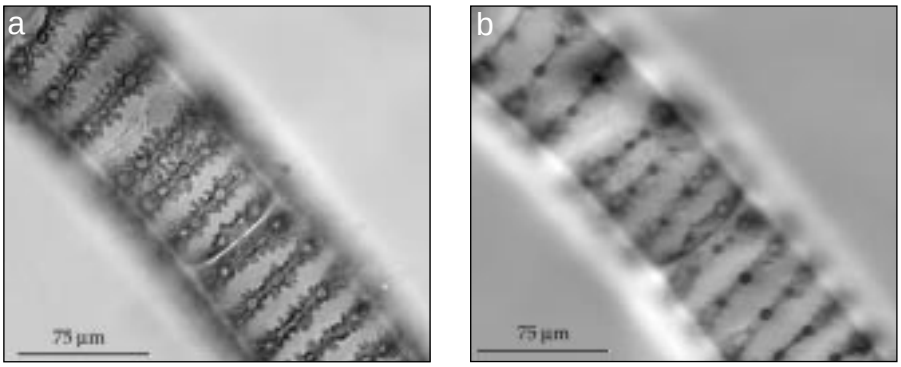

Figure 2.
Spirogyra images. (a) Bright field image of Spirogyra defining the amplitude contrast of specimen (Zeiss Plan-Neofluar 40x 0.75NA air objective). The in focus plane is to the top of the cylindrical Spirogyra and clearly shows the circular pyrenoids in the spiralling bands of chloroplast. The cell wall is seen at the edge of the specimen and the effects of the out-of-focus cell walls at lower planes are visible beyond this. (b) Quantitative phase (optical thickness) of Spirogyra displaying its refractive structure, independent of amplitude effects (computation with QPm, V1.57). The pyrenoids appear dark as they are phase advanced, while the cell walls appear white for phase lag relative to the reference wavefront outside the Spirogyra.

INTERPRETING QUANTITATIVE PHASE IMAGES

An example of a brightfield image of the *Spirogyra* filament is shown in Fig 2a. The smooth, continuous and independent phase information calculated applying the software to an image sequence with 2.0 micrometre steps is shown in Fig 2b. The phase image represents the projection of the sample's refractive structure within the depth of field of the optics. The nature of refraction forces continuity of the phase surface and so is rather more smoothly varying than the absorption and scattering effects imaged in the brightfield image. The high-pass filtering option in the software makes it possible to modify the phase image by highlighting high frequency details in the sample like edges, boundaries and granular structures.

The quantitative phase map of Fig 2b indicates the projection through the pyrenoids is less optically thick and appear dark, while the projection through the cell walls is optically thicker and appear white, when compared with the aqueous media surrounding the *Spirogyra*, which is taken as the reference value and appears mid-grey. The quantitative nature of the software phase has been shown for a well-characterized optical fibre sample [3]. Research is ongoing to characterize this *Spirogyra* sample for quantitative comparison with the software result. Moreover, we can say that given the phase excursion of the sample relative to the aqueous media over the 4.0 micrometre translation range (sample thickness), we can determine the projected refractive index of the various sample components. Alternately, given the refractive index, the optical thickness that the software returns can be used to determine specimen volumes.

QUALITATIVE OPTICAL PHASE VISUALIZATIONS

Conventional optical phase visualization techniques like 'phase', DIC and Hoffman's, convert details of the phase (refractive) structure of the specimen into intensity contrast via interference effects. The standard 'phase' technique makes use of the specimen's actual phase optimized to give the best contrast for small phase shifts. Both DIC and Hoffman's give a measure of the phase gradients across the sample. The phase gradients are represented as shadowing in the image giving the sample a three dimensional appearance.

QPm can reconstruct each of the conventional phase visualization techniques in software based on the amplitude and quantitative phase information initially calculated. Examples of this potential are shown in the images of Figs 3 and 4. A conventional 'phase' image (i.e. Zernike) is shown in Fig 3a, and the software-generated analog in Fig 3b. The analog image has some intensity inversion, but if anything the detail is sharper and organelle definition clearer than the conventional image, a consequence of reduced haloing. In Figs 3c and 3d, we show the conventional and software darkfield images, respectively. A conventional darkfield image presents a quantitative measure of scattering. In this case, the cell walls of the *Spirogyra* scatter more than the chloroplast. The software darkfield presents qualitative scattering detail of the same structures, indicating where scattering will occur, but not to what extent it will occur. Finally, in Figs 4a and 4b, comparison is made between the conventional and software analog DIC, respectively. The calculated analog image is seen to be in excellent agreement with the conventional optical image. We note that the software DIC is generated using unpolarized light. This enables imaging birefringent samples, or when imaging through plastic specimen dishes.

NEW PHASE IMAGES

The software manipulation of QPm opens the possibility of a range of new and specific visualization modalities. One of these overcomes a limitation of conventional phase visualization techniques. This limitation is that generally even "transparent" samples have regions with varying degrees of amplitude contrast. The specialized optics used in conventional phase visualization will incorporate this contrast in the recorded image, a fact generally overlooked in microscopy texts. The interpretation of this intermixing of amplitude and phase contrast is therefore complex.

However, our software allows the microscopist to accurately interpret these mixed

specimens by constructing these conventional phase techniques for phase structure alone, that is, independent of the amplitude detail. An example of this is seen in Fig 4c, where we have produced a DIC image of the *Spirogyra* based only on phase information. This image is independent of the intensity detail seen in Fig 2a. The phase only image provides a qualitative interpretation of the phase structure in the specimen beyond that seen in conventional DIC images and in the software DIC analog (with intermixed intensity) shown in Figs 4a and 4b, respectively, where the phase structure is masked by absorption and scattering.

QUANTITATIVE PHASE IMAGING - NEW INFORMATION AND FUTURE POTENTIAL

As an integrated implementation of quantitative phase imaging, the software package provides a full range of visualizations over a spectrum of experimental configurations, with and without intensity detail. This mode of imaging offers a number of advantages when compared to conventional optical techniques. Most importantly, the requirement for expensive and exacting optical components is obviated. In addition, the capacity to computationally separate phase and amplitude information in the analog images generated significantly extends the analytic options available. Moreover, once captured, the software phase image set represents a virtual specimen that is readily transported, with the software providing a virtual microscope environment.

Quantitative phase imaging can be achieved using other radiations. If through-focal, bright field images can be captured, then it is computationally possible to generate quantitative phase and conventional optical visualizations in situations where currently no practical alternatives exist. Transmission electron microscopy of biological samples is one such possibility [11].

By definition, the quantitative phase is a measure of the optical thickness of the sample, where the optical thickness is the product of the physical thickness and the refractive index of the sample. Since most TEM samples are microtomed to a uniform thickness, the TEM phase image may be interpreted as the projection of the refractive index variation across the specimen. The full potential of non-optical applications for quantitative phase imaging has yet to be explored.

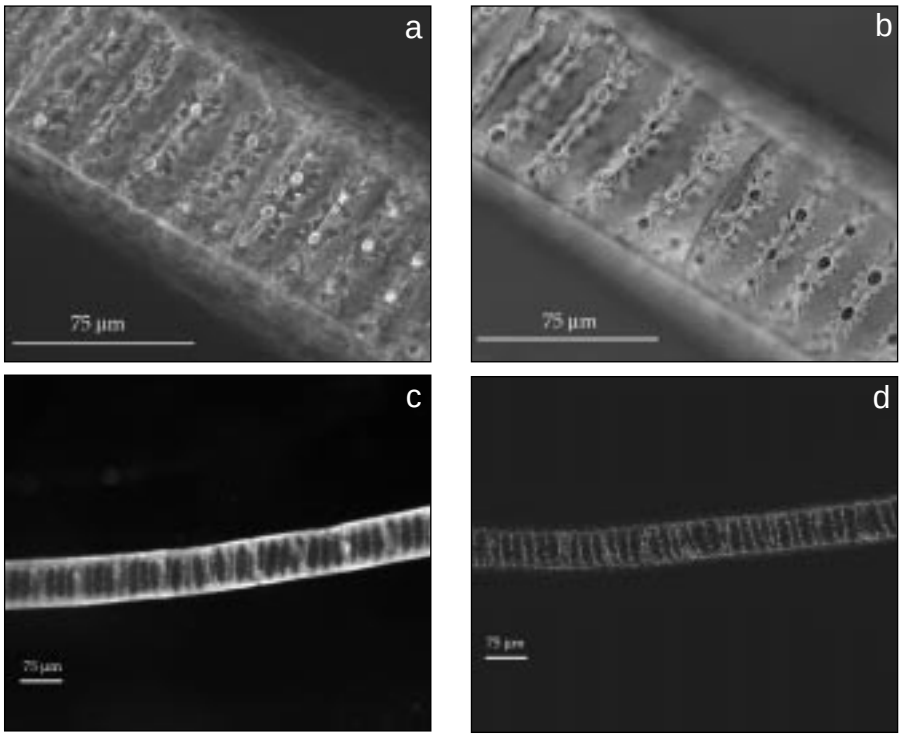

Figure 3:
Spirogyra darkfield and standard phase imaging comparisons (derived from images shown in Figs 2a and 2b). (a) Conventional "phase" image (Zeiss LD-Achroplan Ph 2 40x 0.6Korr air objective). (b) QPm generated "phase" image, showing in places reversed contrast, but generally sharper detail due to less haloing. (c) Conventional dark field image (Zeiss Plan-Neofluar 10x 0.30NA air objective). (d) Software generated dark field image, showing the same outlined features as that of the conventional image.

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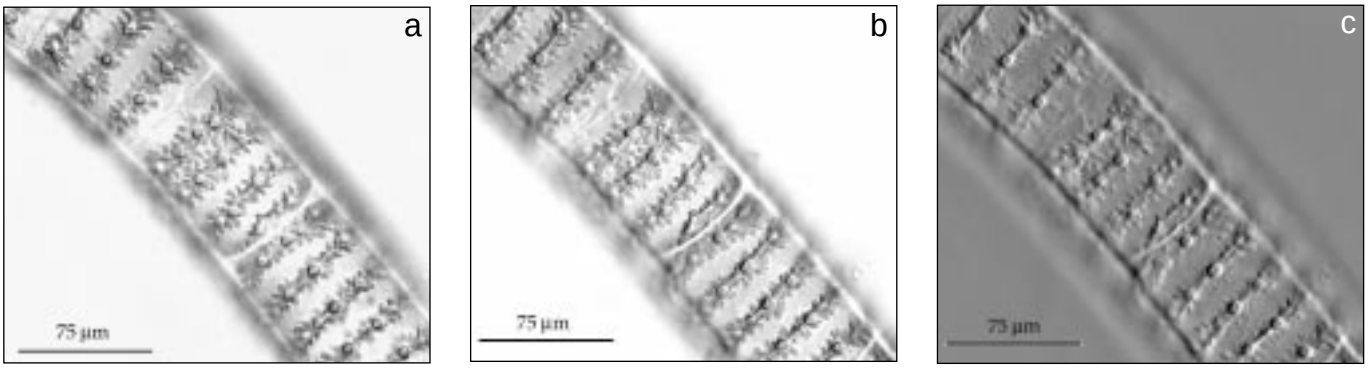

Figure 4:
Comparisons of conventional optically generated Spirogyra DIC image with QPm generated analog images (derived from images shown in Figs 2a and 2b). (a) Conventional DIC image (Zeiss Plan-Neofluar 40x 0.75NA air objective with slider and prism). (b) Software generated DIC analog image incorporating phase and amplitude components showing excellent agreement with the conventional result. (c) Software generated DIC analog image using only the quantitative phase (Fig 2b), that is, independent of the amplitude shown in Fig 2a. This image allows the clear interpretation of phase detail within the sample, as distinct from the information presented in the mixed images of Figs 4a and 4b.