Quantitative Phase Imaging for Transmission Electron Microscopy

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INTRODUCTION

In transmission microscopy, radiation passing through the sample is affected by absorption, scattering, and refraction, changing the amplitude and phase of the transmitted wavefront. These changes to the wavefront carry the structural information about the sample. The image contrast in straightforward ‘brightfield’ microscopy is a result of the scattering or absorption by the sample. However the phase of the wavefield also carries information that is equally or more important. There has therefore been much research devoted to the problem of making visible, and even measuring, the phase of the wavefront. While light microscopy has developed various techniques to achieve phase contrast imaging, such as Zernike phase contrast, Nomarski differential interference contrast (DIC), and Hoffman modulation contrast (HMC). Transmission electron microscopy (TEM) continues to primarily use staining to achieve contrast, moreover comparable phase techniques in TEM are much less convenient.

TEM staining is a chemical process that introduces a highly scattering element, e.g. uranium and lead, into the sample structure. The incident electrons are scattered out of the microscope aperture resulting in strong intensity contrast in the image. However the relationship of the staining process to the sample structure is complex and so has the danger of introducing spurious structure. Furthermore, the stains themselves are often very toxic. The technique described here removes the need to perform such staining.

Phase-contrast techniques have been used for decades in high-resolution biological TEM to expose fine detail in unstained thin samples by two methods - Scherzer focusing and defocus-contrast. These techniques rely upon careful cryomicroscopy TEM methods [1,2] for sample preparation. Thus they preclude the thick resin embedded biological samples typically used in biological TEM studies.

The development of phase imaging methods for TEM that do not rely upon cryomicroscopy has attracted considerable effort, their inherent lack of flexibility has prevented their widespread application to biological samples. For instance, iterative analysis of images obtained at a series of defocus positions [3] works best for periodic samples for which the experimenter has a-priori knowledge. Alternatively, the careful balancing of negative defocus settings provide an intensity proportional to the refractive index [4], but is only suitable for weakly refraction (phase) objects. Finally, phase imaging with electron holography [5] requires a highly coherent field-emission electron source and additional technical apparatus. In spite of these inadequacies, considerable phase information, a consequence of electron refractive effects, has been observed in the wavefront leaving non-periodic biological samples [6].

THE DIRECT MEASUREMENT OF PHASE

A wave is characterized by its frequency, amplitude and phase. The first two are well known as colour and intensity. The third is less familiar. However a spatial variation in phase leads to refraction and this is again a familiar concept. Of course, the effects of refraction are very easy to observe. It has recently been demonstrated that a measurement of refraction - in terms of the variation on intensity with position (think of light being focused by a lens) - enables the phase responsible for this variation to be measured.

This approach to phase measurement - what might be termed propagation-based phase measurement - has a great deal of flexibility compared with more familiar approaches such as interferometry. Interference, in which two coherent waves are overlain and the resulting intensity interference pattern yields information about the phase relationship between one wave and the other, is not well suited to microscopy, principally because of the limitations that flow from the need for coherent radiation.

Propagation-based phase measurement has been applied to measurements of the phase of visible light [7], X-rays [8] and neutrons [9]. It has also been shown to work for electron [10], X-ray [11] and optical microscopy [12-14]. For electrons, the phase of the electron wave is affected by the elemental configuration and the magnetic-field distribution of the specimen. In this article we discuss the application of propagation-based phase measurement to transmission electron microscopy.

THE METHOD

The propagation of a wavefield over a small distance is described by the transport of intensity equation [15]. This is essentially an expression for the conservation of energy for a propagating wave. A key feature of the transport of intensity equation is that a measurement of the intensity of the field, along with its spatial derivative along the optical axis, allows the phase of the wave to be recovered uniquely. In the context of microscopy, this means that a measurement of an in-focus image plus two differentially defocused images either side is sufficient to fully determine the phase of the wavefield. The phase recovery may be performed very rapidly using software commercialised by Iatia Ltd [16].

BIOGRAPHY

Keith A. Nugent is Professor at the School of Physics, University of Melbourne, Australia. Prof. Nugent is a member of the Australian Academy of Science, is one of only 24 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 Quantitative Phase Imaging technology.

ABSTRACT

Although the phase contrast technique is widespread in optical microscopy, phase contrast in transmission electron microscopy (TEM) has not been widely adopted. We show here that a simple method for quantitative TEM phase microscopy is possible. We present examples of TEM phase images of stained and unstained specimens. Moreover, we show how the phase image may then be transformed into image formats, familiar from optical microscopy, which display features that are not visible with conventional TEM images. This technique should permit phase-contrast TEM to be performed almost as readily as phase-contrast optical microscopy.

KEYWORDS

TEM, quantitative phase imaging, phase contrast, DIC, transport of intensity

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This approach is of such generality that it may be applied to any form of microscopy. This application to optical microscopy has been described in an earlier publication [13]. In this paper, we provide further examples beyond those shown in a previous publication [17].

**IMAGE ANALOGUES**

A fully coherent wave is fully specified by its amplitude and phase. As all optical instruments manipulate these two quantities, knowledge of them will permit any other form of phase microscopy to be emulated. There is a wide range of modalities of optical phase imaging. In this article, we will explore the use of the flexible phase imaging described here to emulate these imaging modes in transmission electron microscopy.

**PHASE IMAGING IN THE TEM**

We have developed software [16] to implement quantitative phase imaging for TEM. Conventional brightfield images are imported into the software and processed to define the independent intensity and phase information. As defocus in a TEM also induces both a change in the magnification and a rotation, this processing involves the images being carefully scaled and rotated to bring them into alignment. Additionally, the overall integrated intensities are also scaled to be identical. Under these conditions, the difference between the through-focus images is thus dominated by the phase distribution within the sample. All other processing is performed in the software environment. This includes the generation of analogues to conventional optical phase visualisation techniques including DIC, standard phase, darkfield and HMC, and to generate new imaging visualizations. The software also incorporates a suite of image analysis and manipulation tools.

**RESULTS**

The application of this method to TEM has been well described and its quantitative nature and linearity demonstrated elsewhere [17]. The aim of this section is to present some interesting new images that display the utility of our method.

**Unstained Radula spore**

Sections of unstained Radula (liverwort) spores were imaged at 4,600X using a Philips CM100 transmission electron microscope operated at 80 keV and fitted with a SIS Megaview II Peltier-cooled CCD camera.

These spores were prepared using conventional fixative methods for TEM, followed by dehydration in a graded acetone series followed by infiltration and embedding in resin (LR White, London Resin Co.). Thin (75 - 90 nm) sections were obtained using a Leica Ultracut...
Defocused images were gathered using a nominal defocus of 196 µm and the phase was recovered. The defocus distances required to observe these phase contrast effects are generally larger than those of conventional through-focus series. However, the distances remain small compared to the depth of field of the electron microscope. A theoretical analysis for the method shows that the resolution of this method is not compromised at defocus distances that are small relative to the depth of field [13].

The results are shown in Fig 1. The in-focus image, obtained at a magnification of 4,600X is shown in Fig 1a. The natural contrast in this image is ±10%. An exaggerated phase-contrast image clearly showing the alternating dark and light lines at feature edges within the specimen for a defocus step of 196 µm is shown in Fig 1b. The level of defocus used was comparable to that for which quantitative results were achieved [17].

The data so acquired were used to recover the phase and the result is shown in Fig 1c, and these data were used to generate other modalities. A high-pass frequency filter may be applied to the phase map for further feature enhancement. We thus have a clear demonstration that phase-sensitive TEM is a viable imaging modality using a simple and unmodified electron microscope.

An electron DIC image can be simulated by simply differentiating the complex wavefield in a chosen direction. Conventionally, DIC imaging incorporates intensity information in a complex manner. In this image, we ignore the intensity modulation to generate a phase-only DIC image. The result is shown in Fig 1d.

Note that the DIC image contains a richness of structure not apparent in the transmission image along with fine structure, particularly within the cell, which is not otherwise apparent. A simulated darkfield image of the sample is displayed in Fig 1e, clearly showing the feature edges within the sample, as a conventional darkfield image would.

The QPI technique measures the optical thickness of the sample. The optical thickness is defined by:

\[ \text{optical thickness} = \text{physical thickness} \times \text{refractive index} \]

TEM samples are generally microtomed to uniform thickness so the optical thickness information presented in the phase image depicts the relative electron refractive indices of components within the sample.

Stained Radula spore

Further images of a stained (uranyl acetate and lead citrate) section of a Radula spore imaged at 19,000X, using the same apparatus described earlier, are shown in Fig 2. Figures 2a and 2b show the brightfield and electron optical thickness (phase) images, respectively. Figures 2c and 2d show DIC relief images of the relative optical thicknesses of the samples, without and with the intensity information, respectively. It is clear that the mixing of the intensity in the image (Fig 2d), as happens with conventional (optical) DIC techniques, hides relief structure and makes interpretation confusing.

Mouse stomach

Images of unstained 70-nm resin sections of glutaraldehyde-fixed, osmium-postfixed mouse stomach were captured at a magnification of 30,000X on plate film, using a JEOL.
CONCLUSION

The technique described here is very easy to implement and does not depend on any particular form of TEM. We therefore believe that the approach we have presented here will find widespread use in areas where either staining is undesirable or when staining has the prospect of introducing structure that is not intrinsic to the sample. The ability to image resin-embedded biological samples without staining is a significant new development for all users of electron microscopy, and the demonstration that resin-embedded biological samples show phase contrast yields an important new contrast mechanism whilst avoiding the need to perform cryomicroscopy. Further, we have shown that new imaging modalities more familiar in optical microscopy are possible when phase information is extracted from intensity images. It is hoped that the methodology described here will ultimately permit the observation of features not hitherto seen.

REFERENCES

16. See www.iatia.com.au

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