A Novel Phase-contrast Transmission Electron Microscopy Producing High-contrast Topographic Images of Weak Objects

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Abstract. We report a novel class of transmission electron microscope (TEM), the difference-contrast electron microscope (DTEM), which displays nanostructures of thin specimen objects in a topographical manner. Topography obtained by the difference-contrast develops shadowgraphs in pseudo three-dimension, namely volume-like representation of projected objects as if things are illuminated by light from one direction. The specific optical device to manipulate electron waves for DTEM is the hemicircular π phase-plate, which appears to be quite distinguishable from the Zernike phase plate utilized in Zernike phase-contrast TEM, while both have to be placed onto the back-focal plane of the objective lens. The topographic images obtained with DTEM for ultrathin sections of kidney cells were compared with those obtained with conventional TEM. DTEM confirmed the experimental advantage of high contrast topography by visualizing ultrastructural details inside the cells.

Key words: CTF, difference-contrast, differential-interference-contrast, electron microscopy, phase-contrast, TEM, topography

1. Introduction

Transmission electron microscope is an optical system in which a beam of accelerated electrons is used as an incidence penetrating through specimen objects for generation of images of these objects. Such objects are usually low in amplitude contrast because the change of phases of electron waves is the unique event when electron waves are scattered by the objects. In electron microscopy, the observation of phase changes of waves has been conducted by artificially defocusing the objective lens [1]. This is to change the focal length of the lens as to displace the focal plane from the specimen plane. Such defocusing together with spherical aberration of the objective lens introduces phase shift at the back-focal plane of

the lens. This phase retardation is described through contrast transfer function (CTF) producing changes in image intensity corresponding to changes in object phases. The defocusing approach, however, gives modulation to the signal, namely deterioration of the specimen image quality, by removing information about certain spatial frequencies, around zero in particular, and oscillatingly inverting the sign of higher spatial frequencies [1].

To solve the problem, phase electron microscopy was attempted. One approach was using a phase plate consisting of thin material film of uniform thickness with a tiny hole in the center positioned at back-focal plane of an objective lens [2–5]. Such phase plate is widely known in optics as ‘Zernike phase plate’ [6]. Electron microscope using such phase plate produces improved images of object phases compared to images acquired using defocusing phase contrast. One drawback of such system is the difficulty in operation to align the tiny hole centered in the plate just on the optical axis. A similar implementation is a phase plate consisting of material film with pre-defined radial profile variation and a hole in the center [7]. Such implementation, in addition to optical axis centering difficulties, rises problems with manufacturing of the phase plate and adjustment of pre-determined optimal defocus of the objective lens during observation.

We report the third approach to solve the contrast problem by using new type of phase plate; that is a hemicircular π -phase plate placed onto the back-focal plane of the objective lens.

2. Contrast Transfer Function for Hemicircular π -Phase Plate

The hemicircular π -phase plate works to change the phase by π for only those electrons penetrating through the plate (Figure 1a, b). The wave function through the plate is modulated by a sign-function which takes -1 at the plate hemicircle and 1 at the open hemicircle (Figure 1c). As shown below, such a modulation can completely change the character of the CTF.

For optically weak objects, a disturbance of waves immediately after the object (object wave) is expressed as $I + a(\mathbf{r}) + ib(\mathbf{r})$ ($|a(\mathbf{r})|, |b(\mathbf{r})| \ll 1$); here 1 represents the incidence without scattering, $a(\mathbf{r})$ the object wave absorption (negative), $b(\mathbf{r})$ the change to object wave phase (negative). The CTF induces a phase retardation, $\gamma(\mathbf{k})$, in the form of a multiplier $e^{i\gamma(\mathbf{k})}$ to the diffracted waves, $\delta(\mathbf{k}) + \tilde{a}(\mathbf{k}) + \tilde{b}(\mathbf{k})$. Real ($\cos \gamma(\mathbf{k})$) and imaginary ($\sin \gamma(\mathbf{k})$) components of the multiplier are called the phase- and amplitude-CTF and appear as modulation to the absorption $a(\mathbf{r})$ and the phase change $b(\mathbf{r})$ respectively. Placing a particular phase plate onto the back-focal plane alters the CTF. In Table I CTFs characteristic to two TEM schemes proposed to date are compared with the one proposed in this report, which was designated as DTEM.

Since the phase change of waves is the major source of TEM imaging, the phase-CTF instead of the amplitude one dominantly determines the image contrast. As shown in Table I, aside from the term $i\text{sign}(\mathbf{k})$, DTEM has a feature of

Figure 1. Principle of difference-contrast TEM (DTEM). a. Schematics of DTEM with a hemicircular π -phase plate set onto the back-focal plane. b. Top view of the hemicircular π -phase plate. The central beam passes through the open area close to the edge of the π -phase plate. c. Hemicircular π phase plate inducing sign-function modulation developing in k_x -direction. d. Sine-type contrast transfer function (CTF) developing in k_x -direction, which is multiplied to the absorption of waves. e. Consine-type CTF developing in k_x -direction, which is multiplied to the phase change of waves.

Table I. Contrast Transfer Functions (CTFs) in three classes of TEM [8]

	DTEM	CTEM	PTEM
Phase CTF multiplied to $\tilde{b}(\mathbf{k})$	$[2 \cos \gamma(\mathbf{k})] i \text{sign}(k_x)$	$-2 \sin \gamma(\mathbf{k})$	$2 \cos \gamma(\mathbf{k})$
Absorption CTF multiplied to $\tilde{a}(\mathbf{k})$	$[2 \sin \gamma(\mathbf{k})] i \text{sign}(k_x)$	$2 \cos \gamma(\mathbf{k})$	$2 \sin \gamma(\mathbf{k})$

Here i represents the imaginary unit and $\text{sign}(k_x)$ a sign-function, which defines a function having a constant 1 in the positive domain and -1 in the negative domain along the k_x axis (refer to Figure 1).

$\gamma(\mathbf{k})$: k -dependent phase retardation induced by lens aberrations and defocus.

DTEM: difference-contrast TEM with a hemicircular π -phase plate.

CTEM: conventional (bright-field) TEM without phase plate.

PTEM: phase-contrast TEM with a Zernike phase plate (circular $\pi/2$ -phase plate with a tiny hole in the center).

the phase-CTF completely equivalent to that of the Zernike phase-contrast TEM (PTEM) [5]. As recently proven with PTEM [5], recovery of low frequency components can improve the image contrast. DTEM therefore could add a high contrast to the obtained images. But what is more significant with DTEM is the visual effect of the difference-contrast, which enables to visualize nanostructures in a topographical manner.

The cause why the hemicircular phase plate induces such an effect can be discussed based on the point spread function of DTEM obtained by Fourier transform of the corresponding CTF. To make things transparent, the phase-CTF for DTEM (shown in Figure 1e) was approximated by a simpler function shown in Figure 2a. It is a combination of two square functions as

$$-\Pi(k_x/k_c + \frac{1}{2}) + \Pi(k_x/k_c - \frac{1}{2}),$$

where

$$\Pi(x) = \begin{cases} 1, & -\frac{1}{2} < x < \frac{1}{2} \\ 0, & \text{elsewhere} \end{cases}$$

The cutoff frequency k_c corresponds to the first zero crossing in Figure 1e. The rapid sinusoidal modulation appearing in the frequency region higher than k_c can be treated as bringing about an averaging out effect to signals. The Fourier transform of the approximated function, namely the approximated spread function of DTEM, is given by $\sin(\pi k_c x) \text{sinc}(\pi k_c x)$ (Figure 2b), where $\text{sinc}x = \frac{\sin x}{x}$. Figure 2b shows that the spread function looks like a superposition of two δ functions shifted to each other by k_c with opposite sign. The final image is given by the convolution of the spread function and the original wave function, which naturally leads to a difference contrast in the image and hence the topographic representation.

Figure 2. The simplified CTF for DTEM (a) and the corresponding spread function obtained by its Fourier transform (b).

Above consideration based on the approximation function must be corrected for the actual imaging but the difference feature in the image could be retained.

3. Materials and methods

3.1. PROCESSING OF THE TISSUES

Male Wistar rats (200 g) were fed standard food pellets. Under pentobarbital anesthesia, small pieces of fresh kidney tissues were obtained. They were rapidly frozen by metal contact freezing on the surface of a copper block cooled with liquid nitrogen (-196°C) using an instrument (JFD-RFA, JEOL), and were freeze-substituted with acetone containing 1% (w/v) osmium tetroxide at -80°C in a deep freezer. For freeze substitution tissues were kept in acetone at -80°C for 48 hours, and

then the temperature was gradually raised to -20°C and kept at -20°C for 3 hours, and then the temperature was gradually raised to 20°C . They were transferred to the mixture of acetone/Epoxy resin and embedded in it. Polymerization was done in the sequence of 37°C for 12 hours, 45°C for 12 hours and then 60°C for 12 hours.

3.2. ELECTRON MICROSCOPE

Ultrathin sections ($0.1\ \mu\text{m}$) were made on an ultramicrotome (Oum3, Reichert-Jung) and were collected on formvar-coated copper grids. Without further staining, they were observed in an electron cryo-microscope working at acceleration voltage of 300kV (JEM3100FFC, JEOL), which was modified to accept the π -phase plate.

3.3. PHASE PLATE

The phase plate for DTEM was prepared from an amorphous carbon film, which was made by vacuum evaporation (JEE-400, JEOL) and was gently put onto a $50\ \mu\text{m}$ -diameter objective lens aperture by following the conventional procedure with water surface. The carbon film was adjusted to 60 nm in thickness, which corresponds to π phase shift at 300keV. Part of the film was eccentrically cut out to make a square hole of $20 \times 20\ \mu\text{m}$ by a focused ion-beam machine (JFIB-2300, JEOL).

4. Results

TEM images of ultrathin sections of renal proximal tubular epithelial cells are shown in Figure 3. With conventional electron microscopy (CTEM), the pictures are of low contrast due to high acceleration voltage and no staining, and hence ultrastructures are not clearly observed (the upper row of Figure 3). With DTEM, on the other hand, ultrastructural details such as the cell body, cell organelles could be easily observed, as they are rimmed with the plasma membrane appearing as dark and bright lines, e.g., lysosomes as shown in Figure 3a lower. In the cytoplasm, clusters of ribosomes are observed as obscure structures like clouds with CTEM (Figure 3a upper), but each of ribosomal particles could be well visualized in the DTEM image (Figure 3a lower). Ribosomes observed simply as electron dense particles in CTEM appear as electron-dense particles rimmed with electron-lucent areas in DTEM. This kind of rimming may enable the discrimination of each particle. In the nucleus, the nuclear envelope and its pores are not clearly visualized in CTEM (Figure 3a upper), but they could be well recognized by DTEM (Figure 3a lower). The areas of heterochromatins were also visualized in DTEM (Figure 3a lower).

The two images in Figure 3b show the superiority of DTEM also in the observation of cell membranes. They appear as electron-dense lines with irregularity in

Figure 3. Comparison of images obtained with DTEM and CTEM. a. Electron micrographs of a proximal tubular epithelial cell of rat kidney, rapidly frozen and freeze substituted tissue embedded in a resin. Ultrathin section observed with CTEM (upper) and DTEM (lower). Note that fine structures of various types of cell organelles were clearly visualized with DTEM for samples without conventional staining by heavy elements such as U and Pb. Bar = 0.2 μm . b. Electron micrographs of the same kind of specimen. They were observed with CTEM (upper) and DTEM (lower). Note the presence of granular structures in plasma membranes visualized with DTEM. Bar = 0.2 μm .

CTEM (Figure 3b upper) but they could be observed in DTEM (Figure 3b lower) as electron-dense lines along with electron lucent lines. The close up view indicates that the membrane is composed of tiny granules. These might represent the integral membrane proteins. If this is the case, it may lead to the direct visualization of integrated membrane protein molecules in ultrathin sections.

5. Discussion

The DTEM images shown in Figure 3 clearly illustrate a topographical contrast. The images look similar to images produced by scanning electron microscope. However the 'height' in the DTEM images does not correspond to sample thickness variations (the sample was prepared with uniform thickness) but rather to

the phase delay of the electron wave. This means that areas of higher electron density (heavier material) will be represented as being above their surroundings in the topographical image. If the sample has not uniform thickness then the 'height' in the topographical image will also correspond to the specimen thickness. Such pseudo 3-dimensional representation of the object wave phase seems more natural for the human sense and can simplify the data interpretation.

The topographical image induced by the difference-contrast may show different appearance in image depending on the mutual direction between the target object and the hemicircular plate. The rotation of the hemicircular π -phase plate induces the rotation of the direction the difference is taken, which finally results in an image appearance as if things are illuminated from different angles according to the rotation. Historically this kind of image representation was recognized in the images obtained with the knife-edge aperture (namely the hemicircular stoppling plate) [1]. With a simple consideration, the knife-edge method is proven to be the merge of the two TEMs, CTEM and DTEM [8].

Besides the difference representation, DTEM shows overall much higher contrast compared to CTEM (see Figure 3). This can be helpful for the observation of samples with low contrast, such as non-stained samples or samples observed with higher acceleration voltages. The use of high voltage electron microscopes provides the advantage of observing biological materials with higher resolution. However, to date this merit can not be enjoyed as expected due to its higher transmission power. When ultrathin sections were observed, fine structures can not clearly be observed because of too low contrast of images. The employment of a difference-contrast microscope seems to resolve these problems as illustrated with DTEM images shown in Figure 3.

The unexpectedly high contrast is a particular merit using DTEM, which is thought to be brought about by the recovery of very low spatial frequencies (here up to 0.01 nm^{-1}). Due to various practical difficulties, particularly the severe image deterioration induced by charging of phase plates [1], such a low frequency component were never recorded to date. We could realize such a performance uniquely provided with DTEM during an effort of foundation of phase plate technology [5, 10, 11].

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