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Stroboscopic image capture: Reducing the dose per frame by a factor of 30 does not prevent beam-induced specimen movement in paraffin

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Abstract

Beam-induced specimen movement may be the major factor that limits the quality of high-resolution images of organic specimens. One of the possible measures to improve the situation that was proposed by Henderson and Glaeser [Ultramicroscopy 16 (1985) 139–150], which we refer to here as ''stroboscopic image capture'', is to divide the normal exposure into many successive frames, thus reducing the amount of electron exposure—and possibly the amount of beam-induced movement—per frame. The frames would then be aligned and summed. We have performed preliminary experiments on stroboscopic imaging using a 200-kV electron microscope that was equipped with a high dynamic range Charge-coupled device (CCD) camera for image recording and a liquid N_2-cooled cryoholder. Single-layer paraffin crystals on carbon film were used as a test specimen. The ratio $F(g)/F(0)$ of paraffin reflections, calculated from the images, serves as our criterion for the image quality. In the series that were evaluated, no significant improvement of the $F_{\text{image}}(g)/F_{\text{image}}(0)$ ratio was found, even though the electron exposure per frame was reduced by a factor of 30. A frame-to-frame analysis of image distortions showed that considerable beam-induced movement had still occurred during each frame. In addition, the paraffin crystal lattice was observed to move relative to the supporting carbon film, a fact that cannot be explained as being an electron-optical effect caused by specimen charging. We conclude that a significant further reduction of the dose per frame (than was possible with this CCD detector) will be needed in order to test whether the frame-to-frame changes ultimately become small enough for stroboscopic image capture to show its potential.
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1. Introduction

Beam-induced specimen movement, which may be caused by various factors such as specimen charging, structural rearrangements of the supporting film under the beam, instabilities of the ice or other embedment surrounding the specimen and beam-damage processes to the specimen itself, severely limit the success rate of recording high-resolution data of biological macromolecules with the electron microscope. Quite a number of different measures to improve the situation have been suggested and tried [1–4].

One of the proposed methods, for which we now use the term ''stroboscopic image capture'', divides the exposure that is normally used to record an image into a large number of sub-exposures [3]. In the ideal case, images that are recorded with a fraction of $1/n$th of the full exposure would experience only 1/nth of the beam-induced movement per frame. The effect of beam-induced movement

Abbreviations: ADU, Analog-digital units (same as 'counts', digital output values of the CCD camera); CCD, Charge-coupled device; CC, Cross-correlation; CCF, Cross-correlation function; CTF, (Phase) contrast transfer function; MTF, Modulation transfer function

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should then be at least partially reduced after computational alignment and summation of the fractional-dose images. The reduction in electron exposure is limited, however, by the requirement that alignment of the frames by cross-correlation (CC) must be possible. If the signal-to-noise ratio (SNR) would not be sufficient to distinguish the correct correlation peak in the cross-correlation function (CCF) between pairs of low-dose images, one could use a high-dose image recorded at the end of the series for aligning the low-dose frames in order to extend the degree of dose-fractionation that can be used.

Recent progress in Charge-coupled device (CCD) camera development makes it possible to consider fractionating the dose in this way. Recording stroboscopic image series on photographic film or on older-type CCD cameras is impractical at exposures that are significantly less than is normally used with beam-sensitive specimens, due to the low SNR of these recording media at low electron exposures. More recently, however, high-dynamic range CCD cameras have become available, which provide a significantly higher SNR at low exposures. For instance, for the camera that was used for the experiments described in this paper, the conversion rate was measured as about 165 counts per electron, while the root mean square (rms) background noise was less than 6 counts.

Here we describe first experiments on stroboscopic image capture with such a high-dynamic range CCD camera, using single-layer paraffin crystals on carbon film as a test specimen. As is documented below, the paraffin crystals exhibited rather strong movements even when the exposure per frame was as low as $32\,e^-/nm^2$, which is about 1/30th the exposure usually applied to record a high-resolution image of this kind of sample. This result suggests that the regime in which stroboscopic image capture would become effective for such specimens would first commence at even smaller doses per frame. We show by simulation that image alignment can be performed successfully at even lower exposures than used here. The relatively slow readout of present-day CCDs will then be a limitation, because when recording a stroboscopic image series, the equivalent of a single image would take several minutes. Very likely this limitation will be overcome by a new generation of pixel detectors [5].

2. Materials and methods

2.1. Electron microscopy

The experiments were carried out on a JEM 2100F electron microscope (JEOL, Tokyo, Japan) that was equipped with a field emission gun and a F224HD CCD camera (TVIPS, Gauting, Germany). The microscope was operated at 200 kV accelerating voltage, and the specimen was cooled to $-180\,^{\circ}C$ using a liquid-nitrogen cooled cryoholder (Gatan, Pleasanton, California, USA). As the normal spot-size settings of the microscope did not allow for very low-dose rates, the free lens-control option of the microscope was used to set the C1 condenser to its maximum current. In this way sufficiently low dose rates for the fractionated exposures could be achieved. The CCD camera can be set at two different modes, designated as the "high-capacity" and the "low noise" modes, respectively. For the present studies it was operated in the "low-noise" mode, in which it has a very high SNR. The pixel size of this CCD camera is 24 μm.

Paraffin test specimens were prepared on holey carbon film that was covered with a thin carbon film as described [4]. Before applying the paraffin solution, the carbon-coated grids were heated to ca. 1000 °C under high vacuum for 15 min, in order to enhance the conductivity of the carbon film and to stabilize its structure.

A number of stroboscopic image series were recorded with doses per frame in the range of 30–200 e^-/nm^2. Most of the series were recorded in the form of 1024×1024 images, using the central part of the CCD without binning of pixels, in order to reduce the time per frame. When data were collected in this mode, a beam blanker (above the specimen) was used to limit the exposure time per frame to as little as 200 ms, and the readout time per frame was about 4 s. Specimen drift was therefore negligible during the exposure of individual frames, but successive frames had to be aligned due to the long time that was required to collect a full stroboscopic image series.

2.2. Quantitative evaluation of image-contrast

As in previous work [3,4], the amplitude ratio $F_{image}(g)/F_{image}(0)$ of paraffin reflections was used to characterize the image quality. For evaluating the images the EM and MRC software packages [6,7] were used. The amplitudes $F_{image}(g)$ of the paraffin reflections were calculated in two different ways. Using the EM system software on the raw images, the amplitudes were computed as the square root of the intensity integrated over a 5×5 pixel area centered at the respective peak of the power spectrum, from which the background, determined in a 21×21 pixel area surrounding the peak, had been subtracted. With the MRC software, amplitudes were computed after correcting for image distortions as a vector sum over the 2×2 pixels nearest the reciprocal point, and the background was determined from the perimeter of a 7×7 box surrounding the spot. The spot amplitudes $F_{image}(g)$ were corrected for the MTF of the CCD camera before computing $F_{image}(g)/F_{image}(0)$ ratios. The CTF of the microscope was assumed to be close to 1 for the best images, and no correction was made for the envelope of the CTF and other instrumental imperfections.

2.3. Performance of the CCD camera

The linearity of the CCD and the response time of the microscope beam blanker were checked from an exposure series, with exposure times ranging from 1 ms up to 500 ms. As expected, excellent linearity was found [8]. The response