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Advantages of CCD detectors for de novo three-dimensional structure determination in single-particle electron microscopy

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Abstract

For three-dimensional (3D) structure determination of large macromolecular complexes, single-particle electron cryomicroscopy is considered the method of choice. Within this field, structure determination de novo, as opposed to refinement of known structures, still presents a major challenge, especially for macromolecules without point-group symmetry. This is primarily because of technical issues: one of these is poor image contrast, and another is the often low particle concentration and sample heterogeneity imposed by the practical limits of biochemical purification. In this work, we tested a state-of-the art 4k × 4k charge-coupled device (CCD) detector (TVIPS TemCam-F415) to see whether or not it can contribute to improving the image features that are especially important for structure determination de novo. The present study is therefore focused on a comparison of film and CCD detector in the acquisition of images in the low-to-medium (~10–25 Å) resolution range using a 200 kV electron microscope equipped with field emission gun. For comparison, biological specimens and radiation-insensitive carbon layers were imaged under various conditions to test the image phase transmission, spatial signal-to-noise ratio, visual image quality and power-spectral signal decay for the complete image-processing chain. At all settings of the camera, the phase transmission and spectral signal-to-noise ratio were significantly better on CCD than on film in the low-to-medium resolution range. Thus, the number of particle images needed for initial structure determination is reduced and the overall quality of the initial computed 3D models is improved. However, at high resolution, film is still significantly better than the CCD camera: without binning of the CCD camera and at a magnification of 70k×, film is better beyond 21 Å resolution. With 4-fold binning of the CCD camera and at very high magnification (>300k×) film is still superior beyond 7 Å resolution.

Keywords: CCD; Electron microscopy; Single-particle analysis; Structure

1. Introduction

Projection images of macromolecular complexes obtained by electron cryomicroscopy contain in principle the information needed for the computation of their three-dimensional (3D) structure. Problems in the de novo determination of the 3D structure often arise from uneven angular distribution and a biochemically unavoidable degree of heterogeneity of the sample. In this context, two factors in particular contribute significantly to the difficulty of 3D structure determination. One is loss in image quality (such as poor image contrast), and the other is low particle statistics due to limited concentration of the sample.

In the first steps of de novo single-particle image analysis, the individual particle images have to be aligned properly, to obtain images with improved signal-to-noise ratio (SNR). By the application of multivariate statistical analysis (MSA) followed by classification of images (Frank, 1996; van Heel, 1984; van Heel and Frank, 1981), image classes representing similar views of the molecules become recognisable. The alignment

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algorithms commonly used for this task perform well, even at very low SNR, if reliable reference images (e.g., computed from a 3D structure) are already available (Joyeux and Penczek, 2002). However, for determining a totally unknown 3D structure of a macromolecular complex, reference images computed from an earlier 3D density are not available. In the absence of 3D models, "reference-free" alignment approaches (Dube et al., 1993; Penczek et al., 1992) have to be applied, in order to obtain first particle averages. In the initial image-processing phase, the power of intermediate reference images to drive alignment and classification of the images is obviously much lower than it is in later steps of the image analysis. Therefore, high SNRs are required for successful image-processing. Obviously, the reliability of any 3D structure computed from two-dimensional class averages is strongly dependent on the correctness of the first two-dimensional steps in image-processing. Such class averages normally exhibit structure information up to ~20 Å. Thus, to obtain a reliable 3D structure, the information content of raw images in the resolution range where initial 2D analysis takes place is of great importance. Tests to compare the performance of CCD detectors with photographic film are required—not only in the range of very high resolution (where film is usually better than CCD), but also at low and intermediate spatial frequencies.

Traditionally, conventional silver halide film has been favoured for single-particle data collection, as the procedure is faster and allows the recording of large numbers of particle images in a short time. CCD detectors, in contrast, have the intrinsic disadvantage of low readout speed, coupled with small pixel numbers that limit the speed and efficiency of data collection. So far, CCD detectors have widely been used to record high-resolution electron diffraction patterns of two-dimensional protein crystals (Bullough and Henderson, 1999; Subramaniam et al., 1999), and their applicability for single-particle 3D work (Booth et al., 2004; Stewart et al., 2000; Zhang et al., 2003) and for high tensions up to 400 kV (Brink and Chiu, 1994; Downing and Hendrickson, 1999) has been demonstrated. Currently, CCD cameras are equipped with very efficient polycrystalline phosphor scintillators and cooled to ≈−30 °C, to lower the readout noise (Fan and Ellisman, 2000; Faruqi and Subramaniam, 2000). As a consequence, CCD detectors offer a higher SNR at low resolutions than film does (Booth et al., 2004). Therefore, the use of CCD detectors may nevertheless be worth the extra effort when the image quality of conventional film is inadequate because of low image contrast.

Routinely, the signal decay (Saad et al., 2001) is determined from power spectra of the electron-microscopic images as a first estimate of the quality of signal transmission in terms of a *B* factor. This means that the amplitude information is used to estimate the quality of signal transmission. However, for computerised image-processing, the phase information is of paramount importance, and yet it is not possible to describe the quality of image phases at a certain spatial frequency from the *B* factor without further experimental evidence (Sherman and Chiu, 1997). In our experience, derived from many de novo 3D structure determinations of large (asymmetrical) macromolecular complexes, image alignment together with MSA-based classification is indeed more reliable when a CCD camera is used instead of film (Golas et al., 2005). Another advantage of the CCD camera is the smaller number of particle images needed to obtain first meaningful results. The aim of this work was thus to find an explanation for this practical observation, and to discover why images recorded on a CCD camera are indeed superior for the initial image-processing in single-particle electron cryomicroscopy. Two different approaches were applied to test the reliability of image phase transmission of a 4k × 4k CCD camera and film. First of all, we measured the reliability in image phase transmission by recording series of images from identical areas on a carbon support layer on photographic film or with a CCD camera. After pairwise alignments within each image series, the phase similarity was measured by means of the differential phase residual. The signal from the amorphous sample allowed determination of phase transmission reliability and spectral signal-to-noise ratio (SSNR) in a manner that was independent of radiation damage, but took account of the influence of image alignment. Secondly, images of negatively stained tobacco mosaic virus (TMV) were recorded, and the first steps of single-particle analysis were performed. In this case, the ability of the computer averaging procedure to recover the characteristic helical features in the range of 11.5–23 Å was determined from the power spectra.

2. Materials and methods

2.1. Specimen preparation

For comparable measurements of signal transmission independent of radiation damage, a single carbon film mounted on a copper grid was imaged directly. For specimen preparation of TMV, a solution of TMV in a buffer containing 200 μg/ml virus, 20 mM Hepes and 150 mM NaCl was placed in a Teflon well. The specimen was prepared according to a protocol recently published (Golas et al., 2003). Briefly, a carbon film evaporated onto freshly cleaved mica was floated on the surface of the solution allowing adsorption of virus over 2 min. The carbon film was then transferred to a second well filled with 2% uranyl formate solution and incubated for 2 min. Subsequently, the carbon film with adsorbed particles was attached to a 400 mesh copper grid on which a