Assessing the capabilities of a 4kx4k CCD camera for electron cryo-microscopy at 300kV

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Received 1 May 2006; received in revised form 23 August 2006; accepted 25 August 2006
Available online 22 September 2006

Abstract

CCD cameras have numerous advantages over photographic film for detecting electrons; however the point spread function of these cameras has not been sufficient for single particle data collection to subnanometer resolution with 300 kV microscopes. We have adopted spectral signal to noise ratio (SNR) as a parameter for assessing detector quality for single particle imaging. The robustness of this parameter is confirmed under a variety of experimental conditions. Using this parameter, we demonstrate that the SNR of images of either amorphous carbon film or ice embedded virus particles collected on a new commercially available 4kx4k CCD camera are slightly better than photographic film at low spatial frequency (<1/5 Nyquist frequency), and as good as photographic film out to half of the Nyquist frequency. In addition it is slightly easier to visualize ice embedded particles on this CCD camera than on photographic film. Based on this analysis it is realistic to collect images containing subnanometer resolution data (6–9 Å) using this CCD camera at an effective magnification of ~112 000 on a 300 kV electron microscope.

Keywords: Cryo-EM; CCD camera; 300 kV; Electron cryo-microscopy

1. Introduction

Available detectors in electron microscopy include photographic film, image plates and charge coupled device cameras (CCDs). They differ in the levels of noise, the linearity, the dynamic range, point spread function (PSF) and detective quantum efficiency (DQE) (Samei et al., 1998; Janesick, 2001). Choosing the most appropriate detector for an experiment depends on the resolution goal of the project and the cost-effectiveness in terms of number of micrographs or frames for the entire project. For example, if a single particle project is targeted at 6 Å resolution using electron cryo-microscopy (cryo-EM), the detectable signal that can be extracted from a low dose image at that resolution is the biggest concern. In contrast, data collected in electron diffraction would span many orders of magnitude. In this case, the dynamic range and linearity of the signals become the most important factors in choosing a detector.

Subnanometer resolution structures by cryo-EM are becoming increasingly common (Chiu et al., 2005). The specimen preparation, data collection and image reconstruction have gradually been streamlined for biological end-users. Despite their numerous advantages, the biggest limitation of CCD cameras for the purpose of cryo-EM is the attenuation of high resolution information in images due to the point spread function (PSF) of the CCD camera. In addition, the PSF and its Fourier transform, the modulation transfer function (MTF), are thought to become worse at higher electron accelerating voltage (Downing and Hendrickson, 1999; Meyer and Kirkland, 2000). Recently, a 4kx4k CCD camera (Gatan US4000) has been demonstrated to be feasible for direct data collection with a 200 kV electron microscope and structural determination of biological single particles to subnanometer resolution.
where long $\alpha$-helices of proteins are clearly resolved (Booth et al., 2004; Ludtke et al., 2005; Chiu et al., 2006). It remains unknown if CCD based detectors can be used to collect cryo-EM data for structural determination to subnanometer resolution at higher accelerating voltages. To evaluate the performance of a new CCD detector, a comparison with photographic film provides a benchmark against which new detectors can be measured.

Traditionally, the MTF has been used to describe the attenuation of signal at high spatial frequency. There are a wide variety of techniques used to estimate the MTF for a CCD camera, including, the use of knife edges, interferometer and statistical methods (de Ruijter and Weiss, 1992). Typically, MTF curves are normalized to unity at the origin. The MTF is therefore useful for describing the width of the PSF, but it does not indicate the amount of noise present at a particular spatial frequency. For example if the MTF of a detector decays by 50% at half of the Nyquist frequency, this does not provide any indication if the signal at that frequency can be reliably recovered using standard image processing techniques.

Spectral signal to noise ratio (SNR) has been a common measurement made from images of an amorphous carbon film or an ice embedded single particles (Saad et al., 2001). It is an operational metric which can be influenced not only by the detector but also the microscope performance and the specimen stability. In addition, there are concerns that such a measurement may be dependent on the detector dose, or the size of specimen area to be analyzed. To experimentally establish the robustness of SNR as a detector quality metric this paper describes the effect that different imaging conditions can have on SNR estimation. Images of amorphous carbon are then used to estimate SNR from CCD and photographic film to assess the performance of the current Gatan 4Kx4k CCD camera in a JEM3000SFF operated at 300 kV. Finally SNR calculated from real biological specimens is used to estimate a plausible resolution that can be expected from data collected on such a camera.

2. Materials and methods

2.1. Specimens

Calibration using amorphous carbon and graphitized carbon was done using commercially available grids (Electron Microscopy Services, Ft. Washington, PA). Epsilon15 and T7 bacteriophages were used as biological test specimens. Freshly isolated T7 samples were flash frozen in liquid ethane on washed, electron-beam pre-irradiated (Miyazawa et al., 2003) and glow discharged Quantifoil 400 mesh R2-2 holey carbon grids (Quantifoil Micro Tools GmbH, Jena, Germany). Epsilon15 phage was prepared in the same manner, but a layer of amorphous carbon (~100 Å thick) was deposited on the grid prior to vitrification. Samples were then stored in liquid nitrogen until they could be transferred to the electron cryo-microscope for imaging.

2.2. Cryo-electron microscopy

All images were acquired using a JEM3000SFF electron microscope (JEOL Inc, Tokyo, Japan) operated at 300 kV. This microscope was equipped with the JEOL telemicroscopy software package called FasTEM (JEOL USA Inc, Peabody, MA); with a JEOL top entry cryo-stage operated at liquid helium temperature (4.2 K), and a Gatan US4000 4Kx4K CCD camera (Gatan, Pleasanton CA). The specimen dose rate was estimated from the current density readout in the viewing screen in the microscope. All images were acquired on a Gatan US4000 CCD camera or Kodak SO160 films. The photographic films were developed in full strength D19 at 20°C.

2.3. Collection of images on CCD

JAMES is a custom software package developed at the NCMI for automation of microscopy in JEM2010F microscope (Booth et al., 2004). JAMES has been installed on the JEM3000SSF and was used for data collection. Briefly, this package co-ordinates the communication between the microscope, CCD camera and database (Ludtke et al., 2003) to eliminate the need for dealing with multiple computers and software simultaneously during the data collection. JAMES also provides the optional capability to immediately process the CCD frames collected on the microscope and provide feedback on the quality of the images acquired during a microscopy session.

2.4. Magnification calibration

The magnification of images recorded on the CCD camera were calibrated by calculating the Fourier transform of an image of graphitized carbon (Electron Microscopy Services, Ft. Washington, PA) with diffraction peaks at 1/3.44 Å\(^{-1}\). From the distance, in pixels, that these peaks were located from the origin in the Fourier space, the fold increase of the effective magnification on the CCD camera over that of photographic film was determined to be 1.41 $\times$. In this estimate, we assumed the pixel size of the camera to be 15 μm as specified by the manufacturer. Unless otherwise specified, all magnifications reported in this work are effective magnification based on this measurement.

2.5. Estimating spectral signal to noise ratio

Estimation of the spectral SNR was done as described previously (Booth et al., 2004). Briefly, under low dose conditions an area of thin amorphous carbon film was recorded consecutively on Kodak SO163 film and on the CCD camera. For all images collected on photographic film and CCD, the dosage was held constant on the specimen for both detectors. Images recorded on photographic films were scanned on a Nikon scanner (Nikon Inc., Melville, N.Y.) at a step size of 6.35 μm/pixel. The 2-D power spectrum of each image was estimated by incoherently averag-
ing the indicated number of boxes of 2-D Fourier transforms. Each 2-D Fourier transform was calculated from images of the indicated dimensions. A 1-D power spectrum was calculated by rotationally averaging the 2-D power spectrum. We also define the noise curve to be the baseline that passes through the zeroes in the contrast transfer function (CTF). From the power spectrum and the noise curve, the signal to noise ratio (SNR) as a function of spatial frequency was calculated.

3. Results and discussion

CCD cameras offer a number of significant advantages over photographic film that have been described in great detail. The broad dynamic range, linearity and low level of noise make these devices exceptional detectors for a wide range of scientific applications (Faruqi and Subramaniam, 2000). CCD cameras provide microscopists with immediate quantitative feedback about the quality of both the sample and the data being collected rather than having to wait to develop and then to digitize the film. The most time consuming step with a new specimen for cryo-EM data collection is the search for the proper conditions to collect an optimal number of randomly oriented particles per imaging frame at the appropriate ice thickness. The instant availability of this quantitative information about the specimen and its cryo-preparation makes the initial stage of the project much more efficient.

In electron microscopy, CCD cameras do not require the opening of the camera vacuum for film box exchange which can be a major source of water contaminants in a microscope column even though the photographic films have been well desiccated (Cheng et al., 2006). On microscopes which use a CCD camera as the primary detector and rarely introduce photographic film into the microscope column, we have observed a lengthening of the time in which a sample can be imaged, from several hours to several days without the noticeable buildup of ice contaminants. However, there are some disadvantages to the use of CCD cameras in electron microscopy that until now have made CCD cameras undesirable for cryo-EM studies at subnanometer resolution. The smaller field of view of a CCD camera is of some concern for microscopists. But the PSF of the CCD camera, especially operating on microscopes at 300 kV was a serious limiting factor for subnanometer resolution work in single particle cryo-EM.

3.1. Measuring the sensitivity of SNR to changes in experimental parameters

While estimates of the SNR of CCD cameras for electron microscopy have been described previously on lower voltage microscopes (Zhang et al., 2003; Booth et al., 2004; Sander et al., 2005), an exhaustive evaluation of the robustness of SNR estimate has not been presented. Practically it is relatively easy to evaluate SNR. Typically amorphous carbon is a suitable test specimen for estimating SNR since it has a scattering power similar to biological samples across all spatial frequencies while not showing the same kind of beam sensitivity that real biological samples exhibit. This method for evaluating the noise power spectrum has been shown to give the same result as flat field images collected in the absence of a specimen (Zhang et al., 2003). Nevertheless, we examined critically the effects of different choices of experimental parameters on SNR estimation.

3.2. Effect of cumulative specimen area

Fig. 1 shows two estimates of SNR from the same CCD frame using 2 (512 × 512) boxes and 16 (512 × 512) boxes. The noise level in the estimated SNR curve itself is much higher when only using 2 boxes as compared to 16 boxes, but the magnitude of the SNR peaks is approximately the same from both estimates. From this observation we can conclude that the noise level in the SNR curve itself can change as a function of the number of boxes (i.e. cumulative specimen area) used to calculate SNR as expected when making measurements of a distribution in the presence of noise. However, the relative magnitude of the SNR peaks at different frequencies remains unchanged by changing the number of boxes to calculate SNR. This figure also shows that the total size of the specimen projected onto the detector that is used to calculate SNR does not change the magnitude of the SNR peaks. These observations validate comparison of SNR from images of different cumulative areas such as those collected on different detectors at different effective magnifications.

3.3. Effect of “coherent” vs “incoherent” averaging

The next comparison we made was to calculate the SNR from a single CCD frame, but to vary the way in which the data were used to calculate SNR. Data from a single frame were split into sets of 1, 8, 32 and 128 boxes of 4096, 1024, 512 and 256 pixels, respectively. This example compares the coherent averaging of one larger area of the image to the incoherent averaging of several smaller areas of the same frame. Fig. 2 shows the result of this comparison. The results show that box size and number of boxes does not change the magnitude of the SNR peaks. That is to say, the same SNR in the coherently averaged case is seen as in the incoherently averaged case. This experiment confirms that when comparing detectors the size and number of boxed out areas is not an important factor in estimating SNR.

3.4. Effect of detector dose

To assess the effect of detector dosage on the estimated SNR, we measured the SNR of a single area recorded on the CCD camera with identical beam intensity on the specimen, at two different magnifications. Fig. 3 shows the results of the comparison between SNR calculated at an effective magnification of 83000× and 112000× with different effective detector dosages. The defocus between the two images is slightly
different but the heights of the SNR peaks are independent of the exact magnification or the dosage on the detector. This result firmly establishes the validity of comparing the SNR from images collected under slightly different detector dosages, which is what happens when comparing images collected with different detectors under identical microscope conditions.

There are two caveats to this observation because there are limits to which SNR remain invariant under different magnifications. If the electron microscope is setup at a relatively low effective magnification (for example 55000×), the CTF rings from amorphous carbon image cannot be seen beyond Nyquist frequency defined at that magnification. The measured SNR under this condition will look differently from the same measurement at a higher effective magnification (for example 112000×) in which all the CTF rings may be visible beyond the corresponding Nyquist frequency at the lower magnification. Second, if the measurements are made so that the detector is not operating in its linear range, then...
SNR measurement will not be independent of magnification. This is only important if the detector is operating at extremely low dose (approximately <30 counts/pixel) or an extremely high dosage (>25 000 counts/pixel) (Paul Mooney, personal communication). In our detector, we measured the conversion factor to be ~14 counts/electron. Since our measurements were made within the linear range of the detector response under our experimental conditions, the SNR measurements were independent of the magnification.

3.5. Effect of specimen dose

Finally, we compared the effect of different specimen dosage while holding the other conditions constant. The beam intensity was increased while the dosage on the detector was held at a constant value of 1000 counts/pixel by changing the magnification. Previously we have observed a relationship between the specimen dose and the SNR values estimated for these conditions. This is the first time that this relationship has been verified under carefully controlled conditions. Fig. 4 shows that there is a clear dependence between SNR and dosage on the specimen. Therefore to use SNR as a comparison between detectors one must be extremely careful to keep the dosage on the specimen constant during the experiment. Based on this result, the protocol that was developed to compare detectors ensured that there was no change to the intensity of the beam on the specimen between measurements on different detectors.

Summarizing these results, as expected SNR is a robust measurement tool for assessing the quality of a detector, and if one can ensure that the sample, dosage on the specimen and the CTF are identical between experiments, then SNR can be used as a comparison metric to evaluate the quality of different recording media.

3.6. Comparing SNR between CCD and photographic film at 300 kV

We recorded images of amorphous carbon film on Gatan CCD and Kodak film at two dosage levels (35 and 15 electrons/Å²) and estimated the corresponding SNR (Fig. 5A and B). The optical densities of the photographic film were 0.55 and 0.2, respectively, under these dose and magnification conditions. From these images SNR estimates for both detectors were evaluated for comparison. We have reported the results of this comparison at two different specimen dosage levels corresponding to the range generally used in cryo-EM at 300 kV. Fig. 5A and B shows that the SNR from CCD is clearly greater than the SNR from photographic film up to 1/5 Nyquist frequency (1/13 Å⁻¹ under these imaging conditions). The noise level in the SNR measurement makes it difficult to be certain of any difference between the two detectors beyond half of the Nyquist Frequency (1/5 Å⁻¹ under these imaging conditions) at both specimen dosage levels. Upon careful examination at 15 electrons/Å² under these imaging conditions (Fig. 5B), the SNR from film does appear to be slightly higher than the SNR from CCD at frequency beyond 1/5 Nyquist frequency. However, it is difficult to be absolutely certain whether the difference is real, or is a result of the noise level in the SNR curves. In any case, the differences between the two recording media are relatively small.

We and others have reported similar comparisons at 120 and 200 kV (Zhang et al., 2003; Booth et al., 2004; Sander et al., 2005), and noted that the CCD had enhanced contrast compared to photographic film at low spatial frequency as observed here. The 200 kV data demonstrated that photographic film clearly had a superior SNR than the...
CCD beyond 2/5 Nyquist frequency (Booth et al., 2004). This is the first time that a CCD with 15 μm pixel (i.e. without binning the image) performs similarly to photographic film out to half of the Nyquist frequency.

### 3.7. Evaluating the SNR on CCD images from ice embedded biological specimens

CCD cameras have been successful in collecting data of ice embedded single particles for 3-D reconstructions at relatively low resolution (~14–30 Å) and low accelerating voltage (100–120 kV) (Faruqi and Subramaniam, 2000; Stewart et al., 2000; Rouiller et al., 2001). In addition, we have demonstrated that a CCD camera can be used to acquire data sufficient for a 9 Å reconstruction of single particles at 200 kV (Booth et al., 2004; Ludtke et al., 2005; Chiu et al., 2006). These works illustrate that the smaller field of view of a CCD camera does not limit the ability to collect data for reconstruction at subnanometer resolution. Here we use the method described above to assess the quality of 300 kV image data from ice embedded specimens collected on the 4k×4k CCD camera characterized in this study.

Two biological samples were recorded on the CCD camera to evaluate the SNR of images recorded under low dose conditions. Shown in Fig. 6A is a portion of a CCD frame of ice embedded Epsilon15 bacteriophage taken at 0.75 μm underfocus with a specimen dose of 25 electrons/Å². Even at this defocus level in the presence of a continuous layer of carbon film, the virus particles are clearly visible in these images. The SNR plot calculated from the particle images (Fig. 6C) shows that ~5% signal is present out to 1/5.71 Å⁻¹ (approximately half of the Nyquist frequency). As these images included an amorphous carbon support, the support is contributing to the detected signals.

A second test was done with the ice embedded T7 bacteriophage in the absence of carbon film support. The portion of a CCD frame containing these particles shown in Fig. 6B was taken at 1.1 μm underfocus at a specimen dose of 25 electrons/Å². The particles in this image are very clearly visible, and the SNR calculated from these particle images also displays detectable signal out to half of the Nyquist frequency (Fig. 6D).

Based on the results for two ice embedded single particle specimens, the CCD camera appears to produce images with detectable SNR out to half of the Nyquist frequency. Beyond this point, CTF rings are still visible, but the SNR falls below 5% at the dose level typically used for imaging ice embedded specimens. The SNR of greater than 5% up to and including half of the Nyquist frequency indicates that the optimization of the scintillator for 300 kV has resulted in a superior point spread function that performs better than our previously characterized CCD at 200 kV (Booth et al., 2004). These results correspond to a DQE of 0.07 and an MTF of 0.11 at half of the Nyquist Frequency for this camera (Paul Mooney, unpublished results) based on the characterization method described previously (Mooney, in press).

### 3.8. CCD for subnanometer data collection with cryo-EM specimens

Currently, data that extend beyond 10 Å resolution does not always guarantee that single particle reconstructions will extend to the same level. There are many challenges in the specimen conformation uniformity and image process-
ing that can limit the final reconstruction. It has been noted, however, that the fall-off of detectable signal is an important factor contributing to the total number of particles necessary to solve a structure to subnanometer resolution by cryo-EM (Saad et al., 2001). The fall-off of detectable signal is caused by a variety of different experimental factors and specimen motion. In the past the MTF of the detector was a major contributor to the fall-off of detectable signal when imaging on CCD (Downing and Hendrickson, 1999; Meyer and Kirkland, 2000). Here for the first time, we see that at 300 kV the MTF of the CCD camera may not be the major factor limiting detectable signal to at least half of the Nyquist Frequency. The remaining issue is the limited size of the camera which is still smaller than the photographic films.

A CCD camera with the same performance characteristics but with greater number of pixels has two advantages for biological data collection both related to having more particle images per frame. Greater numbers of particles per frame allows data for a project to be collected faster, and second, it allows one to go up to higher magnification. Estimation of the CTF parameters is essential for subnanometer resolution cryo-EM reconstructions. Collecting higher magnification images is one way to get around the historically poor MTF of CCD cameras. The problem with going to higher magnification is that at some point only a few particles may be imaged per frame. Although the SNR of the particles does not change by having fewer particles (Fig. 1) the CTF parameters can be very difficult to estimate from a CTF curve that is very noisy. With detectable signal around 5 Å at an effective magnification of 112 000 ×, a camera with a larger number of pixels and the same performance characteristics may allow one to replace photographic film for a majority of biological data collection at 300 kV.
Acknowledgments

This research has been supported by NIH Grant P41RR02250 and the Robert Welch Foundation. We thank Peter Weigele and Jonathan King at MIT for providing the virus samples. We thank Paul Mooney of Gatan Inc. for unpublished data characterizing the CCD camera.

References


Fig. 6. Imaging ice embedded single particles with the CCD camera. (A) Image of ice embedded Epsilon15 virus over a layer of amorphous carbon film taken at 0.75 μm underfocus at an effective magnification of 112 000× and a dose of 25 e/Å². (B) Image of ice embedded T7 Phage taken at a defocus of 1.1 μm and the same magnification and dose as in A. (C and D) SNR calculated for the corresponding image of each virus.