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Beam-induced motion of vitrified specimen on holey carbon film

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ABSTRACT

The contrast observed in images of frozen-hydrated biological specimens prepared for electron cryo-microscopy falls significantly short of theoretical predictions. In addition to limits imposed by the current instrumentation, it is widely acknowledged that motion of the specimen during its exposure to the electron beam leads to significant blurring in the recorded images. We have studied the amount and direction of motion of virus particles suspended in thin vitrified ice layers across holes in perforated carbon films using exposure series. Our data show that the particle motion is correlated within patches of 0.3–0.5 μm, indicating that the whole ice layer is moving in a drum-like motion, with accompanying particle rotations of up to a few degrees. Support films with smaller holes, as well as lower electron dose rates tend to reduce beam-induced specimen motion, consistent with a mechanical effect. Finally, analysis of movies showing changes in the specimen during beam exposure show that the specimen moves significantly more at the start of an exposure than towards its end. We show how alignment and averaging of movie frames can be used to restore high-resolution detail in images affected by beam-induced motion.

1. Introduction

Electron cryo-microscopy (cryo-EM) can be used to visualize the three-dimensional (3D) structure of a broad variety of specimens, including two-dimensional (2D) crystals (e.g., Gonen et al., 2004; Henderson et al., 1986), helical specimens (for example, Ge and Zhou, 2011; Miyazawa et al., 1999; Sachse et al., 2007; Yonekura et al., 2003) and isolated (single) particles. In recent years the application of the single particle approach has led to 3D reconstructions of a number of highly symmetrical virus particles at near-atomic resolution (4 Å or better, see Grigorieff and Harrison, 2011) for a recent review). Despite this success, it is commonly acknowledged that contrast in images of vitrified specimens falls significantly short of predicted physical limits (Glaeser, 1999; Henderson, 1995). Physical limits are imposed by the radiolysis of biological molecules caused by the high-energy electron beam which limits the electron dose to 5–10 electrons/Å^2 (Baker et al., 2010; Henderson, 1992, 1995) if high-resolution features are to be preserved. Under idealized conditions, particle images are predicted to contain sufficient signal to obtain a 3D reconstruction at 3 Å resolution by averaging of a few thousand molecular images (Glaeser, 1999; Henderson, 1995). In practice however, the recent reconstructions of particles at near-atomic resolution have required averaging signal from several 100,000 to over 10 million images of subunits or asymmetric units (Grigorieff and Harrison, 2011). The contrast transfer function of the electron microscope, image detector noise and motion in the specimen induced by the incident electron beam all contribute to the loss of contrast in cryo-EM images (for a recent review, see Glaeser and Hall, 2011). The first two issues concern limitations of current instrumentation and are being addressed by technological improvements (Cambie et al., 2007; Danev and Nagayama, 2001; Majorovits et al., 2007; McMullan et al., 2009; Milazzo et al., 2011, 2005; Muller et al., 2010). Beam-induced specimen motion is thought to be caused by the reaction of the specimen to the high-energy electron beam, resulting in a build-up of positive charge on the specimen (Brink et al., 1998) and radiolysis of the sample and vitrified embedding medium (Glaeser, 2008; Glaeser and Taylor, 1978). Charge build-up leads to a weak deflection of the electron beam that can blur the image, especially of tilted samples in which the component of the deflection perpendicular to the beam is not zero (Glaeser and

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Downing, 2004; Henderson, 1992). Radiolysis of the specimen is thought to lead to a build-up of internal pressure as the radiolysis products take up more space than the original molecules (Glaeser, 2008). The mechanical stress is sufficiently high to cause specimen deformations (and ultimately breakdown of the entire fabric – so-called bubbling), again blurring the final image. In a recent study, Glaeser and Henderson (Glaeser et al., 2011) studied the beam-induced motion of paraffin 2D crystals supported by a continuous carbon film and showed that film thicknesses greater than 35 nm significantly reduced the observed motion, thereby improving the fraction of images with strong high-resolution signal. These experiments further corroborate mechanical instability as one of the leading factors allowing beam-induced motion. Unfortunately, the use of a continuous carbon film is often not ideal for non-crystalline single particles as it adds background to an image and can induce preferred particle orientation.

We have recently investigated an imaging protocol in which the electron dose rate was varied, to image single particles embedded in ice over holes in a carbon support film (Chen et al., 2008). These experiments suggested that a lower dose rate allows for a higher total dose before bubbling occurs, but did not clearly demonstrate that beam-induced motion prior to bubbling was reduced. In the present study, we have investigated beam-induced motion by monitoring positions and orientations of rotavirus double-layer particles (DLPs). These particles are very regular and have a molecular mass of 70 MDa, allowing alignment with a reference structure with accuracies of about 0.2 Å and 0.2°, for translational and orientational alignments, respectively (Zhang et al., 2008). We collected exposure series from holes inside perforated carbon films containing rotavirus embedded in ice, varying dose rate and hole size. Changes in the particle orientations between exposures were then taken as an indication for specimen motion. In a second set of experiments, we investigated the timing of the beam-induced motion during exposures by recording movies using a new type of camera, a direct electron detector. Particles visible in individual frames or frame averages were analyzed in terms of their orientational and translational changes during the exposure.

2. Materials and methods

2.1. Sample preparation

Rotavirus DLPs were prepared as described (Street et al., 1982). Three microliters of sample with a concentration of 2.5–4 mg/ml was applied to Quantifoil® or C-flat™ grids and plunge-frozen using either a Gatan CP3 plunger (for all exposure series experiments) or an FEI Vitrobot Mark 2 (for all movie experiments), with a 4 or 6 s blot time and at relative humidity between 65% and 80%. The following grid types were used: Quantifoil® 1.2/1.3 Cu 400 mesh (measured hole size = 1.6 μm; this difference between nominal and measured hole size was observed for all grids in this batch), C-flat™ 0.6/2.0 Cu 400 mesh (measured hole size = 0.6 μm), C-flat™ 1.0/1.0 Cu 400 mesh (measured hole size = 1.0 μm), and C-flat™ 1.2/1.3 Cu 400 mesh (measured hole size = 1.2 μm). The Quantifoil® grids were cleaned prior to their use by immersion in a small amount of ethyl acetate on top of filter paper in a glass Petri dish. Immediately before plunging, all grids were subject to glow discharge for 45 s at 20 mA.

2.2. Electron microscopy – exposure series

Images were collected on a Gatan US4000 4 k × 4 k CCD camera mounted on an FEI TF30 electron microscope operating at 300 kV, and using a side-entry Gatan 626 cryo holder. The calibrated magnification was 49,053, giving a pixel size on the specimen of 3.06 Å. Magnification calibration was performed using a DLP reference structure (Zhang et al., 2008) and maximizing correlation coefficients found by varying the pixel size during *Frealign* (Grigorieff, 2007) runs (see below). We used an underfocus of 2.5–3.5 μm, and an electron dose per exposure of 8 electrons/Å^2. Images in each series were taken about 60 s apart, and occasionally 600 s apart to test if charging was a factor in the observed particle motions (see Section 3). The exposed area on the grid was centered on each hole and held approximately constant at 2.0 μm to ensure that the electron beam made contact with the carbon support film everywhere around holes. Furthermore, an objective aperture was used for all exposures. The ice thickness was measured using holes in the ice layer produced by a focused electron beam with subsequent tilting to 30° (Wright et al., 2006). The ice next to virus particles was determined to be between 800 and 1000 Å thick while it was thinner in the center of holes, with the thinnest ice (500 Å) measured in the center of 1.6 μm holes on Quantifoil® grids.

2.3. Electron microscopy – movies

Images were collected on a Direct Electron DE-12 4 k × 3 k direct electron detector mounted on an FEI TF20 electron microscope operating at 200 kV, and using a side-entry Gatan 626 cryo holder. The calibrated magnification was 17,858, giving a pixel size on the specimen of 3.36 Å. The underfocus was set between 2.5 and 3.5 μm, and the electron dose per frame was 0.5 electrons/Å^2. Movies were recorded at a rate of 40 frames/s. The exposed area was adjusted as above and ice thickness measurements were essentially identical to those measured above.

2.4. Image processing – exposure series

Virus particles were semi-automatically selected from the first image in an exposure series using *e2boxer* from the *EMAN2* processing package (Tang et al., 2007). Micrographs were cross-correlated to each other using the *Spider* processing package (Frank et al., 1996) to determine translational offsets, and particles were selected in subsequent exposures by coordinates updated with the offsets. The defocus for each micrograph was determined using *CTFFIND3* (Mindell and Grigorieff, 2003). Particle images were decimated using 2 × 2 pixel averaging and aligned in an exhaustive search with *Frealign* (Grigorieff, 2007) (Mode = 4 with DANG = 200 and ITMAX = 200 using data between 18 and 300 Å resolution), followed by 10 rounds of refinement (Mode = 1), using a DLP reference structure (Zhang et al., 2008). The rotation angles and axes of reorientations experienced by particles between exposures were determined using the program *Tiltdiff* (Henderson et al., 2011). The rotation axis of most particles was within 0.5° in the image plane. About 6% of the particles were measured to have tilt axes with larger out-of-plane angles. These were excluded from the analysis to eliminate cases were the exhaustive parameter search failed. Histograms of the magnitude of the measured rotation angles were generated with a bin size of 0.1°. The histograms show a clear positive skewness, indicating that a simple average and standard deviation might not be appropriate to characterize the distribution of rotation angles. We used the computer program *distfit* from the *Theseus* package (Theobald and Wuttke, 2006) to test 20 different distributions and selected the Rayleigh distribution as the most appropriate according to the Bayesian information criterion. The Rayleigh distribution (Lalitha and Mishra, 1996) is characterized by a single parameter, λ which indicates the maximum (mode) of the distribution:

$$R(x;\lambda) = \frac{x}{\lambda^2} e^{-x^2/2\lambda^2}. \quad (1)$$

The maximum likelihood estimate of λ^2 is:

$$\lambda^2 = \frac{1}{2N}\sum_{i=1}^{N} x_i^2 \quad (2)$$