Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Structural Biology 151 (2005) 288–297
Journal of Structural Biology
www.elsevier.com/locate/yjsbi

A "flip–flop" rotation stage for routine dual-axis electron cryotomography

Cristina V. Iancu [a,1], Elizabeth R. Wright [a,1], Jordan Benjamin [a], William F. Tivol [a], D. Prabha Dias [a], Gavin E. Murphy [a], Robert C. Morrison [b], J. Bernard Heymann [c], Grant J. Jensen [a,*]

[a] *Division of Biology, California Institute of Technology, 1200 E. California Blvd., Pasadena, CA 91125, USA*
[b] *Gatan (UK), 25 Nuffield Way, Abingdon Oxon OX14 1RL, UK*
[c] *Laboratory of Structural Biology Research, National Institute of Arthritis, Musculoskeletal and Skin Diseases, National Institutes of Health, Bethesda, MD 20892, USA*

Received 3 March 2005; received in revised form 6 June 2005; accepted 6 July 2005
Available online 11 August 2005

Abstract

Electron cryotomography can be used to solve the three-dimensional structures of individual large macromolecules, assemblies, and even small intact cells to medium (~4–8 nm) resolution in a near-native state, but restrictions in the range of accessible views are a major limitation. Here we report on the design, characterization, and demonstration of a new "flip–flop" rotation stage that allows facile and routine collection of two orthogonal tilt-series of cryosamples. Single- and dual-axis tomograms of a variety of samples are compared to illustrate qualitatively the improvement produced by inclusion of the second tilt-series. Exact quantitative expressions are derived for the volume of the remaining "missing pyramid" in reciprocal space. When orthogonal tilt-series are recorded to ±65° in each direction, as this new cryostage permits, only 11% of reciprocal space is left unmeasured. The tomograms suggest that further improvement could be realized, however, through better software to align and merge dual-axis tilt-series of cryosamples.

Keywords: Tomography; Electron microscopy; cryoEM; Dual-axis; Cryoholder; Missing wedge

1. Introduction

The highest resolution technique currently available for three-dimensional structural studies of unique objects is electron tomography, in which a specimen is imaged multiple times in an electron microscope (EM) while being incrementally tilted through a range of views. The method has the potential to couple the exquisite spatial resolution of modern electron microscopes, which in some cases can now be even sub-Angstrom (Batson et al., 2002; Hosokawa et al., 2003), with three-dimensional structure determination through a variety of reconstruction algorithms. Thus, electron tomography is emerging as a powerful new technique in both materials and life science research (Baumeister, 2004; Subramaniam and Milne, 2004; Ziese et al., 2004).

There are several practical limitations for biological samples, however, including radiation damage and the typically restricted range of tilt-angles from which images can be recorded. The tilt-angle limitation arises because most EM samples are thin disks of material approximately 3 mm in diameter and ~30–500 nm thick, and as these samples are incrementally tilted, the depth of material the electron beam must pass through increases as one over the cosine of the tilt-angle. At high tilt-angles, most samples become prohibitively thick.

* Corresponding author. Fax: +1 626 395 5730.
E-mail address: Jensen@caltech.edu (G.J. Jensen).
[1] These authors contributed equally.

1047-8477/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.jsb.2005.07.004

In addition, the edges of standard sample holders block the electron beam at high tilt-angles. Because each projection image provides the amplitudes and phases of just one central section of the specimen's three-dimensional Fourier transform, this data collection constraint translates into a "missing wedge" of information in reciprocal space.

Early in the development of electron tomography, this missing wedge problem was significantly reduced for room temperature samples by collecting two tilt-series about orthogonal axes, thus reducing the missing "wedge" to just a missing "pyramid" (Mastronarde, 1997; Penczek et al., 1995). This procedure was relatively straightforward because room-temperature samples could be removed from the microscope and the specimen holder, manually rotated 90°, and replaced for collection of the second tilt-series without much challenge. Despite complications due to the shrinking and warping of plastic-embedded sections during data collection, dramatic improvements in the quality of the tomograms were realized by inclusion of the second tilt-series, and dual-axis tomography became the recognized standard, best practice (McEwen and Marko, 2001).

Concurrently, various improvements in microscope technology and sample preparation made it possible to image biological materials in a life-like, "frozen-hydrated" state. Such samples are produced by either plunge-freezing thin films (Dubochet et al., 1988) or cryosectioning high-pressure-frozen bulk samples (Al-Amoudi et al., 2004; Hsieh et al., 2002). These developments have made it possible to study the three-dimensional structures of unique objects including even whole cells *in their near-native states* (Baumeister, 2004), and strong efforts are now being invested to maximize resolution and interpretability.

One of the challenges has been the development of a cryostage that allowed facile collection of two perpendicular tilt-series. Because frozen-hydrated samples must always be kept at very low temperatures (standard practice is to maintain better than −160 °C) to prevent ice crystallization, they cannot be simply removed from the microscope, rotated 90° and replaced unless it is done under cold nitrogen gas. This is awkward at best, and is not routinely successful because of the difficulty of fine grid manipulations under these conditions and the frequency of grid contamination. In an attempt to allow grid rotation within the microscope column, the Martinsreid group in collaboration with Gatan modified a standard cryostage and then used it to collect dual-axis tilt-series of at least one frozen-hydrated sample (Nickell et al., 2003), but the stage was not described and seems not to have been used in recent work from the same group (Beck et al., 2004; Cyrklaff et al., 2005; Grünewald et al., 2003; Kürner et al., 2005). A motor-driven tilt-rotation holder (the Gatan CT3500RT) has also been developed for standard side-entry goniometers, but no results of its use in this context are yet available. Here we report on the design, characterization, and experimental benefit of a new "flip–flop" cryorotation stage that allows routine dual-axis cryotomography in the FEI Polara transmission electron microscope, and identify new image processing challenges this type of data presents.

2. The flip–flop rotation stage

The "flip–flop" rotation stage was an experimental product designed and built by Gatan UK (RCM) in consultation with Caltech (GJJ) and others. The prototype was then tested and characterized experimentally at Caltech, as reported below. The stage is a derivative of the new cartridge-based, multispecimen stage system implemented to accommodate liquid-helium sample-cooling in the new Polara series of TEMs from the FEI Company. Frozen-hydrated grids are first clamped into cartridges, and then up to six cartridges at a time are loaded into a multispecimen holder. The holder is sealed, evacuated, mounted on the microscope column, and then opened to the column vacuum. Individual cartridges are picked up with an insertion tool and introduced into the column proper, where they are threaded onto a permanently inserted specimen rod.

The flip–flop rotation stage consists of modified cartridges and a special rotation tool in the multispecimen holder. The modified cartridges house an inner, rotationally mobile cup into which the grid is clamped (Fig. 1A). This mobile cup has two protrusions extending out beyond the edge of the cartridge in opposite directions. The rotation tool is like a two-car garage, in that it can house cartridges in either side, but unlike a garage, it moves over and around the cartridges while they remain fixed (Fig. 1B). When a cartridge is docked in the multispecimen holder, the rotation tool can be pushed over the cartridge (Fig. 1C) in such a way that it catches one of the protrusions on the inner cup and causes the cup to rotate 90°. Depending on which docking position the cartridge occupies in the multispecimen holder, pushing the rotation tool causes the cup to be rotated into either the "flip" position or the "flop" position, 90° away.

Collection of a dual-axis tilt-series proceeds as follows. A cryosample is clamped into the cup of a flip–flop cartridge and loaded into the multispecimen holder. The multispecimen holder is mounted onto the microscope, the flip–flop cartridge is moved to position 5 in the multispecimen holder, and the rotation tool is pushed over the cartridge to ensure the cup begins in the "flip" position. Then the cartridge is introduced into the column, threaded onto the specimen rod, and a suitable specimen is located and imaged through one tilt-series. The cartridge is then retrieved from the column and placed into position 6 of the multispecimen holder, all the while protected by the microscope's column vacuum. The rotation tool is once