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Crystallization Notes

A Script-Assisted Microscopy (SAM) package to improve data acquisition rates on FEI Tecnai electron microscopes equipped with Gatan CCD cameras

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ABSTRACT

High throughput methods of data acquisition are advantageous for cryoelectron microscopy and single particle reconstruction as high-resolution structure determination requires thousands of particle images. We have developed a semi-automated data collection method that utilizes the scripting languages provided by FEI for their Tecnai User Interface (TUI) and by Gatan for their Digital Micrograph package. Our Script-Assisted Microscopy (SAM) method allows for the selection of multiple locations within a low magnification, search mode, micrograph and for subsequent automated imaging of these locations at a higher exposure magnification. The SAM approach permits the user to retain control over the microscope, while streamlining the most repetitive steps of collecting and evaluating micrographs. With SAM, we have found an average of 1000 micrographs can be collected per day on any grid type, either irregular homemade grids or prefabricated grids with regularly spaced holes. This rate of data collection represents a fivefold improvement over our manual collection rates. SAM provides an example of an individually tailored approach to data acquisition utilizing the scripting interfaces provided by the equipment manufacturers. The SAM method has proven valuable for determination of a subnanometer resolution cryoEM structure of DNA-dependent protein kinase catalytic subunit (DNA-PKcs), a 469 kDa protein.

1. Introduction

Cryoelectron microscopy (cryoEM) combined with single particle reconstruction is providing moderate to near atomic resolution structures of a wide variety of biological complexes. The main limiting factor in cryoEM is that biological samples necessitate the use of an extremely low dose of electrons to avoid specimen damage resulting in electron micrographs with low signal-to-noise ratios. Recent work has demonstrated that 4.5 and 3.8 Å resolution structures of the epsilon15 capsid and rotavirus viral protein 6 (VP6) required averaging of the order of 1–6 million repeating units (Jiang et al., 2008; Zhang et al., 2008). Manually collecting large numbers of images is tedious and limited by the amount of data that can be collected from each EM grid before in column ice growth renders the grid unusable (Cheng et al., 2006). Therefore, high throughput data acquisition is extremely useful for achieving high resolution.

For the single particle imaging, several automated data collection packages are available such as Leginon, developed by the National Resource for Automated Molecular Microscopy (NRAMM), which simulates an experienced microscopist (Suloway et al., 2005) in performing the tasks of searching, targeting, exposing, and storing micrographs in a database with limited human interaction. AutoEMation (Lei and Frank, 2005; Zhang et al., 2001; Zhang et al., 2003) also incorporates several levels of automation, allowing automatic or manual grid square and hole selection. However, these packages were designed for commercially available holey carbon grids, which have uniform hole sizes and arrangements. Further, these applications were designed to acquire data at exposure magnifications below 100,000×, in the magnification range suitable for data collection with photographic film (Booth et al., 2004; Lei and Frank, 2005; Stagg et al., 2006; Zhang et al., 2003). In our experience, many samples are best preserved on homemade holey carbon grids. We record micrographs on a CCD camera at relatively high microscope magnifications (>200,000×) in order to oversample the data and allow for computational binning of pixels during image processing. Both the irregular hole arrays of homemade holey grids and the targeting precision (<0.5 μm) necessary for high magnification imaging make data acquisition with the current automated microscopy packages difficult. Due to these limitations, we developed a Script-Assisted Microscopy (SAM) system that augments the FEI and Gatan software to streamline data acquisition and storage.

A modular software platform called JAMES has recently been developed for JEOL microscopes with Gatan CCD cameras (Marsh et al., 2007). JAMES provides a single interface to both a JEOL microscope and a Gatan CCD camera and allows the skilled microscopist to remain in control of most decisions during a microscopy session. We anticipate that since they are modular both SAM and JAMES should be adaptable to other microscopes and CCD cameras.

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1.1. SAM data acquisition strategy

Centering an area of interest manually over the CCD camera for acquisition at high magnification is time consuming and requires precision and good bookkeeping to avoid imaging overlapping regions. Switching between search and exposure modes takes ~15 s in the Tecnai user interface (v2.1.8), and a wait time of ~40 s is necessary to allow the stage to stop drifting after a compustage movement. Evaluation of the recorded high magnification micrograph can include visual assessment of the image, as well as its calculated Fourier transform. If the quality of the micrograph is good, it is saved to disk. This entire manual data collection process can take several minutes per high magnification micrograph. The strategy implemented in SAM is to streamline data acquisition by providing scripting solutions for repetitive events. The SAM approach limits the number of switches between search and exposure modes and minimizes the number of mouse clicks needed to acquire, analyze, and save digital images.

SAM data acquisition begins with the user marking multiple areas of interest with the circle tool in the Digital Micrograph tool kit window on a low magnification, search mode micrograph. Image/beam shift offsets are calculated from the circled positions and applied to the beam to center the selected regions over the camera in exposure mode. This strategy significantly reduces the time required to acquire high magnification digital micrographs, since manual stage movements between the selected areas of a search mode micrograph are eliminated. In addition, only one switch is needed between search and exposure modes for acquisition of all of the targeted regions in a single search mode micrograph. The SAM data acquisition approach has the additional benefits of limiting dose accumulation, simplifying bookkeeping, and ensuring that the same region of a cryo-EM grid is not imaged multiple times at high magnification. The SAM strategy also includes parallel execution of image acquisition and micrograph evaluation. SAM continues to collect high magnification micrographs of additional areas of interest and stores them in system memory, while the user employs another script module to evaluate and save recently collected micrographs.

1.2. Accurate targeting of selected regions at high magnification

Targeting a location with microscope stage movements is estimated to be accurate to ~1 μm (Pulokas et al., 1999; Zhang et al., 2001). Therefore, most current automated EM software packages have implemented image/beam shifts, as we have, as a means to position an area of interest on the submicron scale (Mastronarde, 2005; Suloway et al., 2005). Several steps are necessary before SAM can automatically acquire micrographs with applied image/beam shifts. First, the position of the unshifted beam at the higher magnification of exposure mode without any applied image/beam shifts must be established (Supplementary Fig. 1). This is done by burning a hole in the vitreous ice layer on a test area of the grid using the desired high magnification setting and with the beam spread as it will be during data collection in exposure mode. Then at the lower magnification of search mode, the position of the unshifted beam and the extent of the beam at the higher magnification level should be obvious by the burned area. The user may then mark the boundaries of the unshifted beam position at high magnification using the square tool in the Digital Micrograph tool kit, so that this annotation is different from the targeted regions marked with circles. The coordinates of the square annotation will be used by SAM and are expected to remain the same throughout a data acquisition session.

The SAM Targeting Module automatically calculates vectors from the center of the unshifted beam position to the centers of the targeted regions. Vectors are first calculated within the Digital Micrograph coordinate system and then transformed into image/beam shifts that the Tecnai microscope will need to image the user-selected target regions. Since the Digital Micrograph coordinate system of the camera is not necessarily aligned with the microscope image/beam shift coordinate system, the relationship between the camera X and Y and the image/beam shift X and Y axes must be determined. While the camera X and Y axes are perpendicular, the image/beam shift axes are only close to perpendicular. A mathematic formula for transforming a vector $\vec{S}$ from the camera coordinate system (x,y) to the image/beam shift coordinate system (x',y') is shown in Supplementary Fig. 1. In order to get the SAM package working on a new microscope, the user must determine four parameters (α, β, X_Scale_factor, and Y_Scale_factor), which are used by SAM in the transformation of vectors from the camera coordinate system to the image/beam shift coordinate system. The SAM Calibration section in the Supplementary methods describes in detail how these four parameters may be determined. The user must enter these parameters into the script for the SAM Targeting Module before installing this script into Digital Micrograph.

1.3. SAM Targeting Module

During a data acquisition session with SAM, the user first collects a search mode micrograph using FEI's Tecnai User Interface and Gatan's Digital Micrograph. As described above the user marks regions of interest with the circle annotation tool and the position of the unshifted beam with the square annotation tool. The SAM Targeting Module is started as a custom script within Digital Micrograph and this opens a new window (Supplementary Fig. 2). The Targeting Module automatically obtains the coordinates for all of the annotated regions in the search mode micrograph and creates a text file, called the position file, which contains the image/beam shift coordinates needed for data acquisition of the user-selected regions.

1.4. SAM Acquisition Module

The SAM Acquisition Module reads the information in the position file and passes it to the microscope for data acquisition at high magnification. The Acquisition Module is a standalone Windows program written in Visual Basic and installed on the PC controlling the Tecnai microscope. The SAM Acquisition window (Supplementary Fig. 3) allows the user to browse for the position file saved by the Targeting Module, set the exposure magnification, and specify a delay time. The Acquisition Module will apply this delay both before and after each exposure. We have found that a 5–10 s delay before an exposure is beneficial to allow time for the shifted beam to stabilize. A similar delay, after an exposure, provides a buffer time for synchronizing the command rate with the beam shutter and camera readout. The exposure time used by the Acquisition Module will correspond to that of the last manually collected micrograph. The data acquisition process for all of the user-selected regions in one search mode micrograph begins when the user clicks on the "Automatic Exposure" button. Alternatively, the user may click on "One Exposure Only", which will step through acquisition of the user-selected regions one at a time.

1.5. SAM Evaluation Module

The Evaluation Module generates and displays a binned micrograph and an FFT with a single mouse click (Supplementary Fig. 4).