Asynchronous data acquisition and on-the-fly analysis of dose fractionated cryoEM images by UCSFImage

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Abstract

Newly developed direct electron detection cameras have a high image output frame rate that enables recording dose fractionated image stacks of frozen hydrated biological samples by electron cryomicroscopy (cryoEM). Such novel image acquisition schemes provide opportunities to analyze cryoEM data in ways that were previously impossible. The file size of a dose fractionated image stack is 20–60 times larger than that of a single image. Thus, efficient data acquisition and on-the-fly analysis of a large number of dose-fractionated image stacks become a serious challenge to any cryoEM data acquisition system. We have developed a computer-assisted system, named UCSFImage4, for semi-automated cryo-EM image acquisition that implements an asynchronous data acquisition scheme. This facilitates efficient acquisition, on-the-fly motion correction, and CTF analysis of dose fractionated image stacks with a total time of ~60 s/exposure. Here we report the technical details and configuration of this system.

1. Introduction

In addition to the significantly improved detective quantum efficiency (DQE), all commercially available direct electron detection cameras have high output frame rates, up to 40 frames/s. This feature enables a novel data acquisition approach, termed dose fractionation or movie mode, where an image is readout as a stack of individual subframes recorded during the exposure. This approach enables correction of image blurring caused either by beam-induced motion or thermal and mechanical instability of the specimen holder (Bai et al., 2013; Campbell et al., 2012; Li et al., 2013a), as well as greatly increasing the total electron dose that can be used for imaging frozen hydrated biological samples (Grant and Grigorieff, 2015; Li et al., 2013a; Scheres, 2014).

We previously characterized the K2 Summit™ from Gatan Inc. (Pleasanton, CA) (Li et al., 2013b) and developed a robust motion correction algorithm to correct motion-induced image blurring (Li et al., 2013a). Distinct from the two other commercially available direct detection cameras (from either Direct Electron, LP, San Diego, CA or FEI Company) (Bammes et al., 2012; Milazzo et al., 2011), the K2 Summit was developed specifically to count individual primary electrons striking the camera sensor (Li et al., 2013a,b). Moreover, it can analyze the signal cluster created by each primary electron and centroid the entry point with sub-pixel accuracy, similar to super-resolution methods in light microscopy (Shroff et al., 2007). Thus, the total effective number of pixels is quadrupled to ~58 million.

A standard procedure for collecting low-dose images of frozen hydrated protein samples sequentially steps through three low-dose modes, i.e. Search, Focus and Exposure, to record and save an image to a file system, either mounted on local computer or over the network. After an image is saved, this procedure is repeated. One of the advantages of using a digital camera is the capability of performing on-the-fly image analysis during data acquisition. Thus, some image analysis procedures can also be incorporated into the sequential low-dose image acquisition process once an image is saved. The overall acquisition procedure is well established and efficient (Suloway et al., 2005) when individual images of 4 K × 4 K or even 8 K × 8 K pixels are recorded. However, this simple sequential procedure can become tedious and inefficient when acquiring a large dataset of dose fractionated image stacks, because of the time required to save the very large image stack, especially in super-resolution mode. For example, a dose-fractionated image stack containing 32 gain corrected super-resolution subframes has a file size of ~7.2 gigabytes (GB). It is thus highly desirable to implement procedures that can efficiently coordinate image...
recording, saving, transferring and on-the-fly analysis and also provide a more compact means for storing the raw subframe data.

2. UCSFImage4

We developed a simple to use computer program, named UCSFImage4, to facilitate semi-automated cryo-EM image acquisition. It mimics the manual low-dose data acquisition procedure implemented in the FEI microscope user interface and accepts full intervention from users during data acquisition. It has a flexible interface and can work with different types of cameras, including scintillator-based cameras and direct detection cameras, such as K2 Summit and DE cameras.

Fig. 1A shows the user interface of the program. The main interface of UCSFImage4 runs on a Microsoft Windows computer (XP or newer). It controls an FEI microscope through its TEM server (running on the FEI computer) and FEI scripting interface, and controls the camera through a camera server (Fig. 1B) that is specific for the type of camera being used. It runs on the camera computer and communicates with cameras through plug-ins. For low-dose imaging, it utilizes the low-dose module of Tecnai user interface. Alignment of electron optics and setup of low-dose are all performed on the microscope independent from UCSFImage4. The only calibration that UCSFImage4 requires is to correlate positional information from mouse clicks in Search mode with the stage movement. After such calibration, the user can move the specimen by mouse, pointing and clicking the new position. The User can also use two vectors to define the positions of two neighboring holes of a holey grid with regularly spaced holes (green circles in Fig. 1A), and the program will control the CompuStage to step through each hole collecting low dose images with a pre-defined defocus series. In Focus mode, objective lens current is set to the value corresponding to zero defocus value set in the Tecnai interface. A real time FFT is used for manual or automated focus adjustment. In Exposure mode, an image is recorded with a pre-defined defocus value and saved to a predefined location.

UCSFImage4 has an extensible, modular design supporting a user-defined low-dose data acquisition procedure. All operations of the microscope and camera during a low-dose cycle are grouped and abstracted as sub-steps, such as moving stage, resetting defocus, etc., and named with ID numbers. A user can customize the data acquisition procedure by selecting the sub-steps.

Among all commercial direct detection cameras, the K2 Summit is unique that it is optimized for operation in a single electron counting mode. Despite the very high internal frame rate, minimizing coincidence losses during electron counting requires that K2 images be recorded with a relative low electron dose rate (<10 e⁻/pixel/s) and long exposure time (Li et al., 2013b). To use UCSFImage4 with the K2 camera, we developed a procedure of asynchronous data handling to improve the overall efficiency of data acquisition and on-the-fly image analysis. Together with a number of modules, UCSFImage4 coordinates acquisition of low-dose image stacks and on-the-fly analysis for motion-correction, defocus determination, image quality assessment, and communications between camera, microscope and computers. We also configured and optimized computer hardware to facilitate such asynchronous acquisition and analysis of K2 dose fractionated image stacks from a FEI Tecnai microscope. A typical image stack of 30–50 subframes can be acquired every 60 s. This, coupled with the careful inspection of each hole with some manual intervention leads to the efficient and routine collection of very high quality data sets (Li et al., 2013a; Liang et al., 2015; Liao et al., 2013; Paulsen et al., 2015; Yan et al., 2015). Here we describe its technical details and related hardware and software configurations. While descriptions and discussions of the procedures implemented in UCSFImage4 are based upon using a K2 Summit camera with an FEI microscope, the underlying concepts are quite general and could be applied to other cameras.

3. Asynchronous acquisition of dose-fractionated datasets

As discussed above, recording an image by the K2 Summit with an appropriate dose on specimen, ranging from 30 to 100 e⁻/Å², requires a longer exposure time than typical for a non-counting camera. An image recorded at a lower magnification would require even longer exposure times. For example, using our FEI Tecnai TF30 Polara to reach a total dose of ~34 e⁻/Å² with dose rate of ~10 e⁻/pixel/s on camera, requires ~5 s exposure time to record an image at a magnification of 31k×, and ~13 s at 20k×. With such a long exposure, motion correction is an absolute necessity (Li et al., 2013a). At the maximum output rate of 40 subframes/s, the total number of subframes for an exposure of 5–13 s could be 200–500 or more, although it is more typical to use between 5–10 subframes/s resulting in 25–130 subframes in order to balance the number of subframes in a stack with the needs for successful motion correction (Li et al., 2013a). A single K2 super-resolution subframe has 7420 × 7676 pixels and 8-bit pixel depth prior to gain correction, which converts each pixel to a 32-bit floating-point value. At this precision, each subframe corresponds to a size of ~230 megsabyte (MB). In our current configuration, we typically target the recording of 25–60 subframes per image stack, which corresponds to 6–13 GB of raw data in one exposure.

In order to save both disk I/O time and the disk space, we now save the subframe images prior to gain correction as 8-bit integers and include the gain reference as part of the MRC extended header. This allows the gain corrected floating-point image data to be easily recreated with no loss in precision, while simultaneously increasing transfer speed fourfold and reducing file size 4-fold to 1.6–3.6 GB. The standard MRC format has six modes, 0, 1, 2, 3, 4 and 6, but does not have a mode for 8-bit unsigned integer data type (Crowther et al., 1996). To save the not-gain-corrected subframe images, we added a new mode, 5, for the 8-bit unsigned integer. The binary file structure of MRC file is unchanged. The gain reference is stored as 32-bit float in the extended header of the standard MRC format. Considering that other programs, such as UCSFTomo (Zheng et al., 2007), also use the extended header to store image acquisition information, we put the gain reference immediately after the image acquisition information and until the end of the extended header. Therefore, the size of the extended header will be the total size of the image acquisition information plus that of the gain map. Multiplying the gain reference with the sub-frames converts it to standard MRC format with mode 2, 32-bit real.

To the standard K2 Summit computer configuration we added either an nVidia Tesla C2070 or K10 graphic card to carry out on-the-fly image analysis (see below). Under this basic configuration, when DigitalMicrograph (DM, Gatan Inc.) is used to record a dose-fractionated image stack, subframes are first processed on the K2 Summit processor, followed by being sequentially transferred to RAM on the K2 computer through the 10 Gb network. All data, including all subframes and their summation, are then written to the SSD. Since the entire image stack is transferred first to RAM on the K2 computer before being written to SSD, the time needed to save such an image stack is a major time block that requires optimized coordination with other steps of low-dose image recording cycle. Even with its high I/O bandwidth, it still takes 20–40 s to write an image stack of 1.6–3.6 GB onto the SSD. Together with the time used to transfer an image stack from the K2 processor to the computer, the total time used to transfer and save an image stack is 30–50 s, which is much longer than a single frame acquisition.
In a strict low-dose image acquisition cycle, the microscope setting is switched sequentially between Search, Focus and Exposure modes, followed by recording and saving an image. Prolonged image transferring and saving extends the total time needed to complete a data acquisition cycle. We thus established an asynchronous data acquisition procedure to accelerate this process, that is to overlap the low-dose procedure with transferring data between the K2 processor and the SSD drive in the K2 computer and processing data on the fly (Fig. 2).

Typically in Search and Focus modes, a TV rate camera would provide a live-view of the sample, while in Exposure mode a single exposure of the camera records an image. Because direct detection cameras have a high output frame rate, they can be used for all three modes, greatly simplifying operation and eliminating unnecessary delays. After Exposure, which is usually 5–15 s, the K2 camera returns to the ready state and is immediately switched to live imaging mode. At the same time, the microscope is switched from Exposure to Search mode to start another low-dose imaging cycle, as show in the yellow branch in Fig. 2. Locating the next sample area (Search) and adjusting focus (Focus) takes about 10–20 s. Together with the exposure, the time needed to complete a three-mode low-dose cycle is usually less than 30 s.

In a conventional, serial implementation of transferring, saving and processing the image stacks takes a significantly longer time to complete. Therefore, our main effort is to make this process more efficient. To accomplish this, we save individual subframes to SSD as they become available instead of waiting for the complete stack and overlap saving data to the network with the on-the-fly image analysis (motion correction and CTF analysis), as indicated by the green path in Fig. 2. This significantly shortens the time needed for saving the image stack to SSD, but as the K2 computer no longer calculates the sum of all subframes, this must be calculated separately. However, as data saving is independent from the data acquisition, it can lag behind to an extent limited by the capacity of the SSD (1 TB in our configuration). Even with the overlaps, the total saving and processing time is longer than that strictly required for the three-mode low-dose cycle (usually less than 30 s). To keep the two branches synchronized, we usually set a 10–20 s delay between focus and exposure modes, which also helps to stabilize the mechanical motion of the microscope stage. With such an asynchronous acquisition, a low-dose acquisition cycle can be limited to within one minute, corresponding to at least 60 stacks per hour. This is actually significantly faster than many fully automated acquisition systems.

4. Coordinating data acquisition and on-the-fly image analysis

UCSFImage4 implements the asynchronous data acquisition flow of Fig. 2 and uses three external modules (image binning, motion correction and CTF determination) for on-the-fly processing of dose-fractionated data (Fig. 3 and Supplementary Fig. 1). All modules run as individual programs and communicate through internet-sockets used by most computers so that the modules can be easily distributed to different computers with different operating systems. All the data on different computers are shared between different operating systems, either by SAMBA between Microsoft Windows and Linux or by NFS for Linux computers.

On-the-fly image analysis must be fast enough to maintain synchronization with the data acquisition with the minimal added delay. To speedup this process, we often use 2 x 2 binned images instead of full size super-resolution images, although all image registration is applied to the super-resolution images. While image binning can be calculated by averaging adjacent pixels, such averaging causes signal attenuation at high frequency by a sinc function and aliasing of higher-frequency signals into the lower frequency binned images. We instead crop the central m/b x n/b pixels from the fast Fourier transform (FFT) of the full sized raw image of m x n pixels (b = binning ratio), followed by an inverse Fourier transform. Such a b-fold binned image keeps exactly the same Fourier components in the Fourier space within the cropped region (resolution) as the original raw image. A potential problem is that the cropping in Fourier space is equivalent to convolving a sinc function with every pixel in real space. Thus, any bad pixels with very large or small intensity values will influence a large adjacent area. To minimize the influences of bad pixels, flat fielding references must be prepared carefully previous
The bad pixels must be corrected before the binning. We also carry out all image analysis using general-purpose graphic processing units (GPUs). In both the Fourier-space image binning and motion correction, the Fourier transform is the major computation that is calculated several hundred times per image stack and occupies \( \frac{90}{C24} \) computing time. However, on a typical CPU using the fastest FFTW3 library, a single direction FFT on a super-resolution frame (7420 \( \times 7676 \) pixels) will take \( \frac{3}{C24} - 6 \) s. The total processing time for one stack would then be at least 20 min, which is too slow not only for on-the-fly but even for practical off-line processing. In our configuration, we used nVidia GPUs and CUDA (nVidia) to accelerate the FFT and all other computations. GPUs have previously been used to accelerate many aspects of cryoEM image processing (Li et al., 2010; Zhang et al., 2010). They are particularly powerful for calculating FFTs; with a 7420 \( \times 7676 \) pixel FFT taking only \( \frac{0.05}{C24} \) s on a GTX680 GPU with 1536 cores. Image binning and motion correction are usually more than 60-fold faster on a GPU than with a comparable CPU version. For this purpose, we installed a Tesla C2070 graphic processor on the K2 computer, so that image binning and motion correction can be immediately carried out once the frame stack is ready. Both procedures can be completed in 20–30 s for a stack with 30–40 frames. This is sufficiently fast to give immediate feedback to user before the next image is recorded. The on-the-fly motion correction can also be done on a separated Linux workstation with one or more powerful GPU cards if the network connection between the GPU computer and K2 computer can support a data transfer better than 400–500 MB/s (discussed below). Note, that in our experience the higher-end computational (Tesla) cards are considerably more robust than the equivalent commodity display versions.

5. Conclusions and discussion

We described here an asynchronous data acquisition procedure that is routinely being used for efficient acquisition of large numbers of dose fractionated image stacks recorded on a K2 camera connected to an FEI Tecnai microscope. This procedure enables us to collect \( \sim 600 \) image stacks within 10–12 h. It can also be easily adapted for other cameras capable of recording dose fractionated image stacks. Note that the asynchronous data acquisition procedure described here was designed for improving efficiency of acquiring dose-fractionated electron-counting images. While the procedure also works for charge integration cameras, the improvement of data acquisition efficiency may not be as significant as for a counting camera.

As the total number of subframes increases, the bottleneck of this asynchronous data acquisition becomes transferring data between the K2 computer and an external processing or storage server. Transfer rates of at least 400–500 MB/s seem to be necessary to make the asynchronous procedure smooth, requiring both high speed networking and high performance RAID storage systems. For the network, 10 Gb Ethernet is a minimal requirement, although QFSP or InfiniBand with more than 40 Gb bandwidth or the combination of multiple 10 Gb channels is preferable. To optimize the on-the-fly image processing, we configured a separate computer with a RAID0 of 10 SSDs to increase the storage bandwidth to be comparable to the high-speed network linking to the K2 computer and two nVidia Tesla K10 graphic cards for on-the-fly image processing. By sharing its storage with K2 computer, the large stack file will be directly written from K2 computer’s memory in order to avoid the bandwidth and capacity bottleneck of the local storage on K2 computer.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jsb.2015.09.003.

References


