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Multi-resolution correlative focused ion beam scanning electron microscopy: Applications to cell biology

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ABSTRACT

Efficient correlative imaging of small targets within large fields is a central problem in cell biology. Here, we demonstrate a series of technical advances in focused ion beam scanning electron microscopy (FIB–SEM) to address this issue. We report increases in the speed, robustness and automation of the process, and achieve consistent *z* slice thickness of ~3 nm. We introduce "keyframe imaging" as a new approach to simultaneously image large fields of view and obtain high-resolution 3D images of targeted sub-volumes. We demonstrate application of these advances to image post-fusion cytoplasmic intermediates of the HIV core. Using fluorescently labeled cell membranes, proteins and HIV cores, we first produce a "target map" of an HIV infected cell by fluorescence microscopy. We then generate a correlated 3D EM volume of the entire cell as well as high-resolution 3D images of individual HIV cores, achieving correlative imaging across a volume scale of 10^9 in a single automated experimental run.

1. Introduction

Current technologies such as X-ray crystallography, NMR spectroscopy, and cryo-electron microscopy are capable of determining the 3D structure of biological specimens ranging in size from small protein complexes to intact viruses at resolutions of ~0.2–10 nm. Likewise, cells and tissues can be routinely imaged with light microscopy at 3D resolutions as high as ~100 nm. However, there is no satisfactory technology that can efficiently image an entire cell in 3D at both light and electron microscopic resolution, where regions of interest identified by fluorescence microscopy can subsequently be imaged by electron microscopy at resolutions of ~10 nm or better. Traditionally, 3D ultrastructure has been obtained using serial section – transmission electron microscopy (TEM), a technique that is labor intensive, prone to artifacts and suffers from low resolution along the axis perpendicular to the cutting plane. Serial block face scanning electron microscopy provides a more automated solution, combining microtomy and imaging using a scanning electron microscope (SEM) (Briggman et al., 2011; Denk and Horstmann, 2004; Shu et al., 2011). Nevertheless, this technology still suffers from the problems of lower resolution along the *z*-axis axis and artifacts arising from manual sectioning. In focused ion beam scanning electron microscopy (FIB–SEM, also referred to as ion abrasion scanning electron microscopy or IA-SEM) (Drobne et al., 2008; Heymann et al., 2009; Knott et al., 2008), resin-embedded samples are subjected to an iterative process of milling (slicing) with a focused ion beam (typically gallium), followed by imaging by the SEM. This generates a stack of 2D EM images that is computationally converted to a 3D ultrastructural volume of the sample. FIB–SEM has been used recently to describe various structures in biology, including virus–cell interactions, mammalian and non-mammalian cells and tissue architecture at 3D resolutions approaching 10 nm (Drobne, 2013; Heymann et al., 2006; Hildebrand et al., 2009; Murphy et al., 2011).

While FIB–SEM is already a powerful tool in the arsenal for 3D biological imaging using electron microscopy, important limitations remain in the use of this nascent technology. First, the speed of data collection is limited by the time required for sequential SEM image acquisition and ion beam milling: imaging entire cellular volumes at 3D resolutions of 10 nm can take many days for completion. Second, the resolution of imaging is generally anisotropic and while effective pixel sizes as low as 3 nm can be

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obtained in the *xy* plane, obtaining the same resolution reproducibly in the *z*-direction has proven problematic. Finally, a problem that plagues imaging techniques in general is the forced trade-off between resolution and size. Small areas can be viewed at the highest possible resolution afforded by the technique, but larger fields of view must either be imaged at low resolution, or at high resolution by inefficient procedures such as tiling (Schroeder et al., 2011). These limitations in turn result in significant challenges for applications requiring true correlative imaging, where nanoscale objects are located using fluorescence microscopy, and the ultrastructure of the same regions are then determined by 3D electron microscopy.

In order to address these issues, we have used a new "keyframe" imaging strategy that enables point-and-click high resolution 3D ultrastructural imaging of local regions of interest (ROIs), while also obtaining lower resolution ultrastructural information of the entire field of view. The technical advances that enable this are the following: (i) we target high-resolution imaging to only those regions which are of interest within a given field of view, (ii) we speed up the rate of acquisition by milling and imaging simultaneously instead of consecutively, (iii) we correct for drift in 3D, allowing the recording of images that consistently achieve resolutions better than 10 nm in all three planes and (iv) we combine this with light microscopy to identify regions of interest within a given volume. We show that all of these goals can be achieved for imaging both bacterial and mammalian cells using a commercially available microscope and demonstrate that a small nanoscale object such as a 100 nm-sized HIV core can be localized and imaged at 3D resolutions better than 10 nm within the cytoplasm of a 40 μm wide mammalian cell (Supplementary Figure S1).

2. Results and Discussion

In Fig. 1a, we show a schematic of the keyframe method of imaging. In this example, the field of view encompasses several adherent and non-adherent cells, and the high resolution ROI is the cell–cell contact zone. As we progress with imaging, we obtain two sets of images. One set, named the "keyframe", includes the entire field of view imaged rapidly at a resolution of ~20 nm in the imaging (*xy*) plane, and ~100 nm in the *z* direction. Between successive keyframe images, we obtain images of the ROI at much higher pixel sampling, typically ranging from 3–10 nm in the *x*, *y*, and *z* directions. This is achieved without changing magnification, thus the optics are kept stable throughout the run. The ROIs can be altered in real time: in an experiment examining cell–cell contacts between dendritic cells and adherent carcinoma cells, a circular ROI was set up based on an initial keyframe (Fig. 1b and d); however, subsequent keyframes revealed a new area of interest (Fig. 1c). The shape of the high resolution ROI being imaged was then altered during the course of the run to now include the new area, allowing it to be imaged at high resolution in 3D (Fig. 1e). To further increase the speed of data collection on the FIB–SEM, we addressed the use of the two beams. In conventional FIB–SEM imaging, resin milling by the FIB and image acquisition by the SEM are performed sequentially. By synchronously imaging a face and milling it, with both beams constantly turned on, we can achieve significant gains in speed, reducing the time required for data acquisition by ~2-fold, with little or no loss in image quality (Supplementary Figure S2).

We next extended the technology to achieve isotropic resolution in all three planes. Pixel sizes of ~3 nm are possible in the *xy* plane, but to achieve this level of resolution in the *z*-direction, it is necessary to measure and maintain ion beam stability over the entire imaging session. Although the beam can mill in increments as small as 0.1 nm, the stability of ion beam milling can be thwarted by thermal and mechanical fluctuations that cause slow drifts as well as unpredictable movements as large as ±200 nm or more in all 3 directions. To measure and correct for this drift, we deposited an approximately 1 μm thick, patterned platinum–carbon double layer on the top surface of the volume. Because the cross sections of the patterns (Supplementary Figure S3) are visible in each successive image, they can be used to dynamically correct for drift in all three axes, and serve as markers for automated focus and stigmation correction. Using this approach we were able to obtain images at a consistent *z*-spacing of ~3 nm, correcting for changes in drift, focus and stigmation during data collection runs spanning several days. To test this system, we collected a stack of 2D images of the sporulating bacteria *Bacillus subtilis*. These bacterial forespores are surrounded by a proteinaceous coat, comprising at least 20 different crosslinked polypeptides and organized into several distinct layers, ranging from <20 nm to several hundred nanometers. Shown in Fig. 2a is a plot of the slice thickness for an image acquisition run of a large volume of resin-embedded bacteria, where the user-defined pixel sampling was 3 × 3 × 3 nm. The average measured thickness of the *z* slices for this run lasting nearly 24 h (excluding the earliest period during system stabilization) was 3.25 nm, with a standard deviation of 0.54 nm, demonstrating an exquisite control of the FIB over long periods of time, even with soft biological material. The data are presented in a 3D volume resampled with 5 nm-sized voxels, establishing that the quality of the 3D image is similar in all three viewing planes (Fig. 2b). At least 5 coat layers surrounding the spore core are resolved in each plane (Fig. 2c–e), and features as small as ~20 nm proximal to the central core (arrowheads) are resolved. Image slices through representative bacteria from the original *xy*, *xz*, and *yz* planes, without resampling and aligning, are shown in Supplementary Figure S4. The fine features of the spore coat layers are visible in all 3 axes. Importantly, the ability to image a given volume in 3D with isotropic pixel sampling means that the image resolution of any given sub-region (in this case, individual bacteria within the pellet) is independent of its orientation within the larger volume.

Because specific, intracellular protein labeling is difficult to achieve for FIB–SEM, it is useful to correlate fluorescence microscopy with this technique. In previous correlative FIB–SEM work, we reported methods for image registration that enabled the detection of a single 100–200 nm-sized spherically shaped fluorescently labeled virus particle in contact with a T cell, where the entire region was imaged at resolutions of ~20 nm (Murphy et al., 2011). Given the improvements in imaging described above, we applied our methods for keyframe imaging and isotropic resolution to correlative imaging of the HIV core. The HIV core is a conically shaped entity filled with the packaged genome of the HIV virus, which enters cells during the process of infection. To test whether we could localize and image HIV cores following their entry into a target cell, we infected cells expressing YFP-labeled TRIM5α with mCherry labeled fluorescent HIV. TRIM5α is a critical host-specific retroviral restriction factor (Perron et al., 2004; Stremlau et al., 2004) known to associate with the HIV capsid (Ganser-Pornillos et al., 2011). When proteasome function is inhibited, TRIM5α forms "TRIM bodies" that capture apparently intact HIV cores in the cytoplasm of infected cells (Anderson et al., 2006; Campbell et al., 2007). Locating and visualizing, in a single imaging session, the mCherry labeled 100 nm-sized HIV core, the YFP-labeled TRIM5α, as well as the entire cell, which is many orders of magnitude larger, is a stringent test of the keyframe imaging methodology. Starting from a fluorescence microscopic image that allowed clear visualization of both HIV and TRIM5α (Fig. 3a), we carried out 3D imaging of the entire cell, collecting keyframe images at lower resolution (*xyz* pixels of 12, 12 and 120 nm respectively, Supplementary Movie M1), combined with targeted imaging of the regions