Journal of Structural Biology 176 (2011) 268–278

Contents lists available at SciVerse ScienceDirect

Journal of Structural Biology

journal homepage: www.elsevier.com/locate/yjsbi

Correlative 3D imaging of whole mammalian cells with light and electron microscopy

Gavin E. Murphy [a,1], Kedar Narayan [a,1], Bradley C. Lowekamp [b], Lisa M. Hartnell [a], Jurgen A.W. Heymann [a], Jing Fu [a,2], Sriram Subramaniam [a,*]

[a] Laboratory of Cell Biology, Center for Cancer Research, National Cancer Institute, NIH, Bethesda, MD 20892, USA
[b] Office of High Performance Computing and Communications, National Library of Medicine, NIH, Bethesda, MD 20814, USA

ARTICLE INFO

Article history:
Received 14 February 2011
Received in revised form 22 August 2011
Accepted 25 August 2011
Available online 1 September 2011

Keywords:
Ion-abrasion scanning electron microscopy
Focused ion beam scanning electron microscopy
Correlative light electron microscopy
T cells
HIV

ABSTRACT

We report methodological advances that extend the current capabilities of ion-abrasion scanning electron microscopy (IA-SEM), also known as focused ion beam scanning electron microscopy, a newly emerging technology for high resolution imaging of large biological specimens in 3D. We establish protocols that enable the routine generation of 3D image stacks of entire plastic-embedded mammalian cells by IA-SEM at resolutions of ~10–20 nm at high contrast and with minimal artifacts from the focused ion beam. We build on these advances by describing a detailed approach for carrying out correlative live confocal microscopy and IA-SEM on the same cells. Finally, we demonstrate that by combining correlative imaging with newly developed tools for automated image processing, small 100 nm-sized entities such as HIV-1 or gold beads can be localized in SEM image stacks of whole mammalian cells. We anticipate that these methods will add to the arsenal of tools available for investigating mechanisms underlying host-pathogen interactions, and more generally, the 3D subcellular architecture of mammalian cells and tissues.

1. Introduction

Ion-abrasion scanning electron microscopy (IA-SEM), also referred to as focused ion beam scanning electron microscopy, is a technology for 3D imaging that has been adapted recently to biology for the 3D imaging of large mammalian cells and tissues at nanoscale resolution (Heymann et al., 2009; Subramaniam, 2005). In this approach to 3D imaging, a gallium ion beam is used to abrade material progressively from the surface of the specimen and a scanning electron beam is used to generate an image from each newly exposed surface, producing a stack of images that provide a 3D representation of the imaged volume. With IA-SEM, we and others have previously reconstructed 3D images of yeast cells (Heymann et al., 2006), melanoma cells (Heymann et al., 2009), diatoms (Hildebrand et al., 2009), HIV-1-infected macrophages (Bennett et al., 2009), T cell-dendritic cell synapses (Felts et al., 2010), HeLa cells (Lucas et al., 2008), mouse neurons and arteries (Hekking et al., 2009; Knott et al., 2008; Merchan-Perez et al., 2009; Siskova et al., 2009), zebrafish vasculature (Armer et al., 2009) and diseased liver mitochondria (Murphy et al., 2010). Serial ultramicrotomy inside the chamber of the microscope in conjunction with block-face imaging has also been used as an alternative approach to generate 3D images of cells and tissues, although at lower resolutions in the direction orthogonal to the plane of sectioning (Denk and Horstmann, 2004; Shu et al., 2011). While the presently available methods have been useful to provide new, and sometimes unexpected insights into subcellular architecture in each of these cases, there is a need to develop additional tools to enhance the use of IA-SEM for investigating cellular mechanisms, especially in the context of correlative microscopy.

Recently, a variety of methods have been developed that combine TEM with light and fluorescence microscopy to allow correlative imaging using these two methods (Gaietta et al., 2002). These include approaches that involve hardware changes in the specimen chamber of the electron microscope to allow simultaneous fluorescence imaging (Agronskaia et al., 2008), as well as methods that are based on visualization of fluorescence in plunge-frozen samples with subsequent evaluation using cryo-electron microscopy (Sartori et al., 2007). Another approach used fluorescence *en bloc* light microscopy of thin-sectioned, high-pressure frozen and freeze-substituted eukaryotic cells and correlated it to the corresponding tomograms (Kukulski et al., 2011). One limitation of these methods is that since TEM imaging is restricted to regions of the sample that are less than 0.5 μm thick, correlative imaging is restricted to either a section from the cell, or to the thin edges of whole mammalian cells (Jimenez et al., 2010; Sartori et al.,

* Corresponding author. Fax: +1 301 480 3834.
E-mail address: ss1@nih.gov (S. Subramaniam).
[1] Both authors contributed equally to this work.
[2] Present address: Department of Mechanical and Aerospace Engineering, Monash University, Clayton, Australia.

1047-8477/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.jsb.2011.08.013

2007). Since IA-SEM is capable of providing 3D images of whole mammalian cells, it is desirable to develop techniques that allow the imaging of entire cells first using live confocal microscopy and then by IA-SEM to obtain a correlated SEM image stack.

Once a SEM image stack is generated, methods that enable rapid feature identification in these large data sets must be developed. Image stacks with a lateral sampling of ~3 nm even from single mammalian cells can be very large (~1 gigabyte or larger) and pose significant technical challenges for mining information. While methods to carry out automated segmentation of these SEM image stacks involve procedures similar to those used in segmenting tomograms, locating small features of interest such as viruses poses an even greater challenge because they represent a much smaller fraction of the overall volume imaged. Feature identification using template-based approaches or machine learning (Narasimha et al., 2009) can provide a starting point for these analyses, but typically result in a very high frequency of detection of false positives because of the complexity and the low signal-to-noise ratios inherent to IA-SEM. One possible solution to high-fidelity, automated, feature recognition is to label the feature of interest with a fluorescent tag; then, the signal from the correlated confocal image volume could be used to identify the feature of interest while simultaneously excluding false positives generated by automatic segmentation.

Here, we present progress towards each of the problems identified above to further streamline the use of IA-SEM in biological imaging. We provide a perspective on the relative merits of different staining methods and identify conditions that produce the most useful image contrast. We present a method that enables regions identified in a fluorescence microscopic image to be easily identified and imaged with IA-SEM. We also present novel image processing tools for registration of light and electron microscopic images, and automated tools for segmentation to extract selected features such as 100 nm-sized viruses out of the large volume of mammalian cells. Together, these developments provide additional enhancements to tools for 3D imaging of mammalian cells at nanoscale resolution.

2. Materials and methods

2.1. Cells, viruses and particles

For the staining screen, dendritic cells and CD4 + T cells were generated and purified from peripheral blood, and then incubated with HIV-1 BaL viruses as published previously (Felts et al., 2010). For the correlative experiment, the CD4 + T cell line H9 was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. The cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 100 U/mL Penicillin/Streptomycin and were >95% viable at the time of experiment. Infectious HIV-1 BaL viruses purified from supernatants of infected SupT1 CCR5 Cl.30 cells via sucrose gradients were treated with Alexa488-maleimide and Aldrithiol-2 (Sigma–Aldrich) to yield fluorescent viruses (kind gift from Dr. Jeff Lifson, SAIC Frederick). 100 nm-sized gold beads (BBI Inc., Cardiff, UK) were used as control particles because they are similar in size to HIV and are clearly visible by EM, and they were incubated with irrelevant fluorescent antibodies before the experiment.

2.2. High-pressure freezing and freeze substitution

H9 T cells mixed with 100 nm-sized gold beads and AT-2 treated HIV-1 BaL were placed into a solution of 4% paraformaldehyde (PFA) and 20% bovine serum albumin (BSA) for 20 min. The cells were spun down gently and then 0.7 μL of the pellet were added to a gold-covered planchette 0.1 μm deep and 1.5 μm wide (Leica Microsystems, Vienna, Austria). The planchette was then frozen in an EMPACT high-pressure freezer (Leica Microsystems, Vienna, Austria). Several staining solutions were tried. Those that produced poor staining or preservation lacked PFA and included conditions that used (i) the OTO method, (ii) BSA with only 0.5% uranyl acetate, (iii) osmium, uranyl acetate and glutaraldehyde with and without BSA and (iv) osmium, uranyl acetate, glutaraldehyde and water. The best staining solution, a mixture of 1% osmium tetroxide, 0.5% uranyl acetate and 0.5% glutaraldehyde in acetone, was prepared and dispensed into capsules (Leica Microsystems catalog #702738, Vienna, Austria) held in a metal block and then frozen in liquid nitrogen. The frozen staining solution was transferred to a styrofoam container filled with liquid nitrogen. The planchettes were then transferred to the styrofoam container and each planchette was placed face-up into a capsule. The capsule-containing block was then transferred to the chamber of a freeze substitution device (Leica Microsystems) maintained at −90 °C, and a warming protocol was initiated. The sample window was covered with aluminum foil to prevent destruction of the stain. The samples were maintained at −90 °C for 7 h; warmed at a rate of 2 °C/h for 32.5 h to −25 °C; maintained at −25 °C for 12 h, then warmed at a rate of 2 °C for 12.5 h to 0 °C, and maintained at 0 °C for 3 h. The block was then placed on ice and the staining solution was removed and washed with cold acetone every 40 min (three times). After the last wash, the acetone was replaced with a solution containing 1:2 ratio of Embed-812 resin to acetone and stored at room temperature for 4 h. The solution was finally replaced with a solution containing resin and acetone (2:1 ratio) for 2.5 h. The planchettes were then removed from the capsules and placed face-up in inverted Beem capsules (EMS) whose pointed bottoms had been removed. Following the addition of 0.5 ml of pure resin, the Beem capsules were maintained at room temperature overnight, followed by baking in a 60 °C oven for 24 h. The Beem capsules were subsequently removed from the hardened resin. Resin around the embedded planchette was removed with a Weck razor blade, and the block was dipped into liquid nitrogen, warmed and then dipped again, in order to dislodge the planchette gently from the resin. The planchette was removed and the sample-containing region of the resin block was shaped into a pyramid. The top was subsequently smoothed with an ultramicrotome and the top 2 mm of the block was then attached to a SEM stub. A comparison of data obtained from high pressure frozen, freeze substituted and corresponding chemically fixed T cells is shown in Fig. S1. Because no substantive differences were observed in this preliminary comparison, chemically fixed cells were used in all of the studies reported here.

2.3. Staining screen

A flow chart depicting the various staining protocols is included (Fig. S2). Briefly, dendritic and T cells isolated from peripheral blood mononuclear cells (PBMCs) were fixed with 2.5% glutaraldehyde in 100 mM sodium cacodylate buffer, pH 7.4, for 4 h and stored at 4 °C overnight. Cells were washed three times for 5 min each in 100 mM sodium cacodylate buffer, pH 7.4. The sample was then fixed and stained with 2% osmium tetroxide, which itself was reduced with 1.5% potassium ferrocyanide, in cacodylate buffer for 1 h. *En bloc* staining (i.e., additional staining using chemicals added post-fixation but pre-embedding) was tested using potassium permanganate, uranyl acetate, ammonium molybdate and phosphotungstic acid. For this purpose, the osmium-treated samples were divided into two aliquots, one with, and one without 1% potassium permanganate added for 4 h in the dark at 4 °C. The two groups were then each divided into four additional groups to be stained with (i) saturated uranyl acetate in 70% methanol, (ii)