Multi-Signal Characterization of Biological Structures at Low-Voltage Using STEM-in-SEM

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The imaging of soft and biological materials, or "soft electron microscopy", focuses on the ability to characterize materials with high contrast at low enough electron doses to avoid sample damage [1]. Preparation and imaging techniques are driven by the length scale of the structures, requirements for sample preservation, and resolution necessary to identify key morphological features. For example, the solving of single proteins requires the resolution of cryo-electron microscopy with direct electron detectors, while the imaging of cells and tissues often uses room temperature fixation, staining and sectioning.

While biological structures are most commonly imaged using transmission electron microscopy (TEM), we previously reported the use of scanning transmission electron microscopy (STEM) in a scanning electron microscope (STEM-in-SEM) for the high-throughput characterization of protein assemblies [2]. The STEM-in-SEM technique shows promise for a range of biological structures, both stained and unstained; the low electron voltages available in SEM have the potential to improve contrast because of the increased scattering cross-section [3]. In addition to improvements in contrast with transmission imaging, the multitude of other signals available in SEM can provide a holistic structural characterization for biological materials. These signals include secondary electrons, backscattered electrons (BSE) and spectroscopic methods such as energy dispersive x-ray spectroscopy (EDS), among others.

This work focuses on the bridge between nano- and microscale biological structures, specifically osmium-stained cells treated with iron oxide magnetic nanostructures (MNS) [4]. STEM-in-SEM and BSE imaging are used to identify contrast originating from different sources in a high-throughput, accessible technique.

THP-1 macrophages were treated with low-density lipoprotein (LDL) and Fe_3O_4 magnetic nanostructures with a method similar to Singh *et al.* [5]. Cells were fixed using glutaraldehyde and paraformaldehyde, stained with osmium tetroxide, dehydrated through a series of increasing ethanol concentrations, embedded in resin, and sectioned using ultramicrotomy. High angle annular dark field (HAADF) STEM images were acquired on a Hitachi HD-2300A dedicated STEM at 200 kV, bright field (BF) STEM images were acquired using STEM-in-SEM at 30 kV on a Hitachi SU8030 with an insertable STEM detector and dedicated TEM grid holder, and BSE images were acquired on a JEOL JSM-7900FLV-SEM at 30 kV.

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Figure 1 compares a more traditional HAADF STEM image at 200 kV using a dedicated STEM (Figure 1a) with BF STEM at 30 kV using STEM-in-SEM (Figure 1b-c). HAADF STEM imaging is highly dependent on atomic number contrast and therefore clearly shows clustered iron oxide MNS. The nanoparticles are also resolvable in 30 kV BF STEM-in-SEM images with sufficient contrast. Although contrast enhancement is theoretically expected at lower voltages, contrast in this case depends more on the influence of the osmium stain and MNS. Knife marks from sectioning are visible particularly at 200 kV; this would inhibit the practical use of these images with traditional HAADF STEM. Overall, STEM-in-SEM using BF detection provides sufficient contrast to distinguish important features of the cell and high enough resolution to image MNS and their integration in the cell.

In addition to transmission imaging, SEM instruments can acquire a multitude of signals that benefit biological imaging. Of particular interest for samples stained with heavy metals is BSE imaging; similarly to HAADF STEM, BSE signal is highly dependent on atomic number for elastic scattering. Figure 2 highlights the benefits of BSE imaging on stained cells. First, promising cell clusters are identified at low magnification across many grid squares using the large field of view in SEM (Figure 2a). As shown in Figure 2b, BSE contrast identifies areas with high concentrations of osmium; the treatment of cells with LDL is expected to produce globules of fat, which draw OsO_4 more than other parts of the cell due to the compound's attraction to unsaturated bonds [6]. Figure 2c suggests that MNS clusters are distinguishable in contrast and morphology from osmium staining, and this characterization revealed that they are primarily localized in regions with a higher concentration of osmium. Osmium has a higher atomic number than iron and would be expected to provide brighter contrast; however, the concentration of osmium atoms is quite low compared to that of iron atoms in MNS, so the MNS clusters appear brighter in these images. One significant advantage of BSE imaging over HAADF STEM is that BSE signal is produced from the deep in the section and largely ignores surface features. This means that the knife marks on the surface of the section that were visible with STEM do not interfere with image quality when using BSE.

This work demonstrates both STEM-in-SEM and BSE imaging, often underutilized in the field of biological characterization, for the structural interpretation of stained and sectioned cells. While this analysis highlights one important SEM modality for biological imaging, spectroscopic techniques such as EDS or wavelength-dispersive spectroscopy (WDS) are invaluable to identify elemental localization. The high-throughput, high-contrast and relatively low-cost nature of SEM make it a prime choice for the characterization of a range of biological, polymeric or hybrid structures.
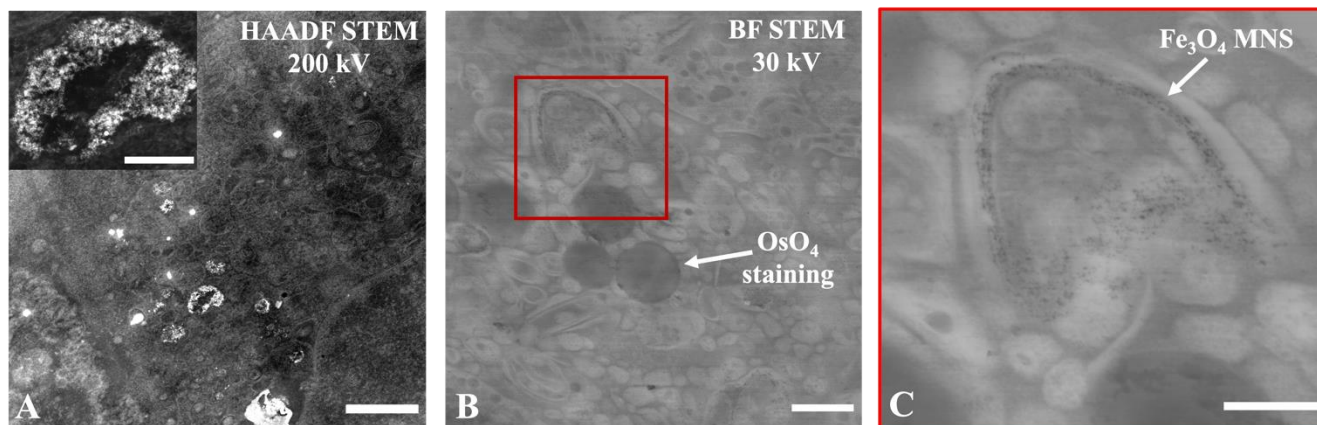
Figure 1. a) HAADF STEM image of MNS-treated, sectioned, and osmium-stained cell at 200 kV with a 62-330 mrad collection angle. Scale bars 0.5 μm and 0.1 μm (inset). b) BF STEM at 30 kV using STEM-in-SEM. Scale bar 1 μm. c) BF STEM image of area boxed in b highlighting iron oxide MNS. Scale bar 0.5 μm.
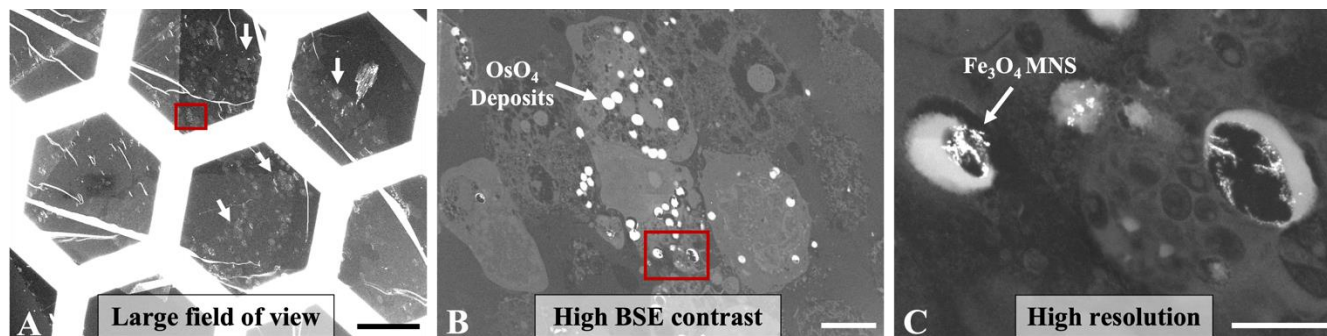

Figure 2. Backscattered electron images in SEM at 30kV highlighting a) the large field of view in SEM, where arrows indicate promising clusters of cells, b) high BSE contrast with clear osmium deposits and c) high resolution showing iron oxide MNS. Scale bars: a) 100 μm, b) 5 μm, c) 2 μm.

References:

[1] V Dravid. Proceedings of Microscopy and Microanalysis (2020), p. 2014-2015. doi:10.1017/S1431927620020140.

[2] K Parker, V Dravid et al., Proceedings of Microscopy and Microanalysis (2020), p. 1026-1028. doi: 10.1017/S1431927620016712.

[3] C Hall in "Introduction to Electron Microscopy" (McGraw-Hill Book Company, New York 1953).

[4] V Nandwana et al., ACS Applied Materials & Interfaces **8(11)** (2016), p. 6953-6961. doi: 10.1021/acsami.6b01377.

[5] A Singh et al., ACS Nano **13(9)** (2019), p. 10301-10311. doi: 10.1021/acsnano.9b03727.

[6] V Wigglesworth. Proceedings of the Royal Society of London. Series B - Biological Sciences (1957), p.185-199. doi: 10.1098/RSPB.1957.0043.

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