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Dual Beam FIB for Imaging, Nano-sectioning and Sample Preparation of Spores: Initial Results

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Abstract

Results from the first use of Focused Ion Beam (FIB) technology to section *Bacillus* spores at LLNL in a dual-beam (electron and ion) instrument is presented and discussed. With the use of a dual-beam instrument, high resolution imaging of single spores using low voltage scanning electron microscopy followed by FIB sectioning, SEM imaging of internal structure of the same spore is demonstrated to be possible. Additionally, FIB is shown to be able to precisely micro-machine spores thus potentially facilitating micro-scale experiments on single spores.

Introduction

Dual-beam FIB is a rapidly maturing technology. Dual-beam systems combine high resolution scanning electron microscopy (SEM) imaging with a high-resolution focused ion beam imaging and machining. Modern day instruments are designed such that specimens can be positioned at the intersection point of the electron and ion beam to an accuracy of much less than 1µm. This allows nearly simultaneous imaging and nano-sectioning and machining of single isolated spores.

A salient goal of our work with spores is to develop analytical procedures that can reveal information about the sophistication and knowledge of the producer, the geographical origin of the component materials used to produce the agent, and the time of manufacture relative to release or discovery. Of great importance are techniques that can analyze individual agent particles and thus are applicable to trace samples that may contain only a few agent particles, or are contaminated by debris unrelated to the agent or its production. In short, this report will demonstrate that dual beam FIB/SEM technology and techniques are not only potentially important for finding, observing and characterizing single spores but may also facilitate unique characterization and experimentation by other techniques via the specialized preparative sectioning and micro-machining.

Experimental

The spores used for these proof-of-principle dual-beam applications are *Bacillus subtilis* var. *niger*. The typical shape of the spores in this sample is oval with a length of ≈1.2 µm and a diameter of ≈0.6µm as determined by SEM. Thespores were dispersed on a Si substrate and dried on a hot plate in air at a temperature of approximately ??X^{o}C. The dual-beam instrument used is a Model DB235 Strata from FEI Co. This work was performed at the FEI Co. demonstration facility in Hillsborough, Oregon. Imaging with the electron beam was done at voltages between 2 and 5 keV to minimize charging and electron beam damage. Sectioning and machining was performed with a Ga ion beam column operating at 30 keV and a beam current of 1 pa. *In situ* Pt deposition coating of approximately 5 nm was used to eliminate any final charging effects.

Results

Initial imaging of the spores with secondary electrons (SEM mode) was straightforward. Figure 1a is a typical lower magnification image of individual and groupings of spores that are uncoated. The effects of charging can clearly be seen in the image as dark horizontal streaks. The basic shape of the spores is observed to be oval

with a typical length of ≈1.2μm and a diameter of ≈0.6μm. The surface texture ranges from being fairly smooth to have a range of texture or roughness. Some spores appear to have debris attached to them (comments from Woods on this). Figure 2 shows a much higher magnification of an individual spore from a region in Figure 1.

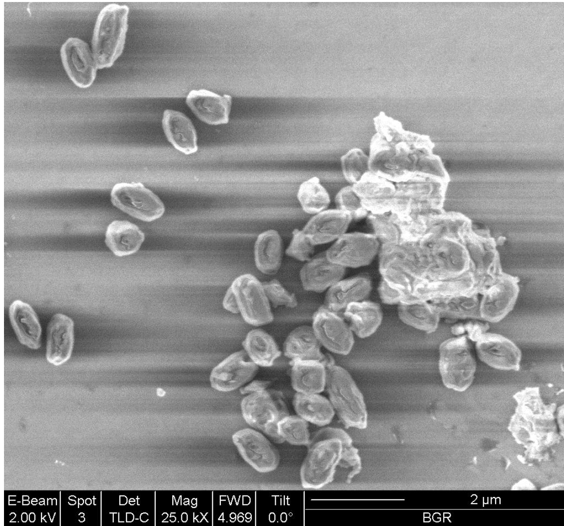


Figure 1. SEM image of uncoated spores. Figure 2. Enlarged region of Figure 1.

In a dual beam FIB either the electron beam or the ion beam can be utilized in conjunction with a deposition gas to locally deposit a number of materials. In order to eliminate the effects of charging, a thin layer of Pt metal was ion beam deposited over a small region containing a number of spores. Figure 3 is an example of a SEM mode image of coated spores, while Figure 4 shows a higher magnification view of a portion of Fig. 3.

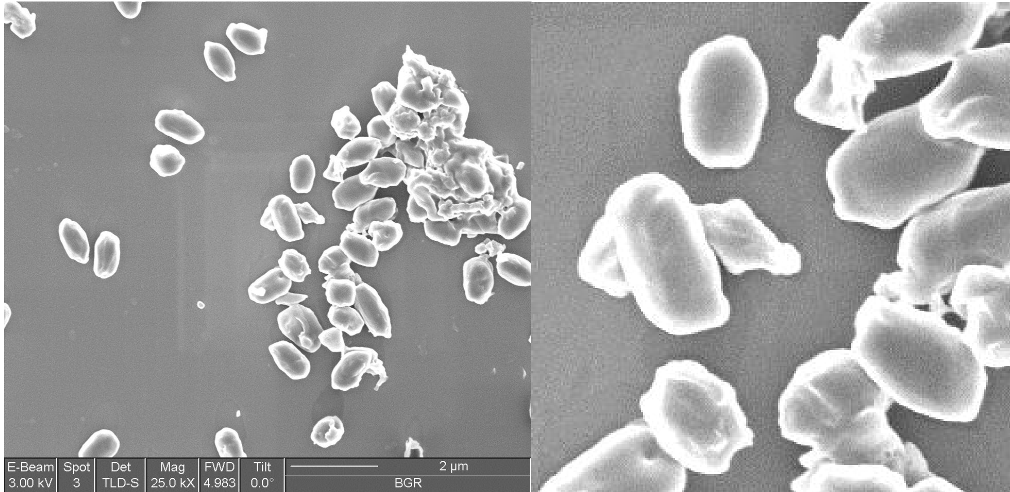


Figure 3. SEM image of Pt coated spores. Figure 4. Enlarged region of Figure 3.

It can be seen that the charging effects have been eliminated with the addition of a 5 nm Pt coating, thus greatly increasing the image quality. However, the Pt coating has had a smoothing effect to the higher frequency surface structures. Figures 1 and 3 are identical regions and were acquired using the same imaging conditions. Additionally, Figures 2 and 4 are cropped and equally enlarged region from Figures 1 and 3, respectively.

Figure 5 is a high magnification SEM image of a single isolated and coated spore. This single spore was then sectioned in half using a 1pa, 30 keV Ga ion beam. The cutting time to remove one half of the specimen, down to the Si substrate, was approximately 60 seconds.

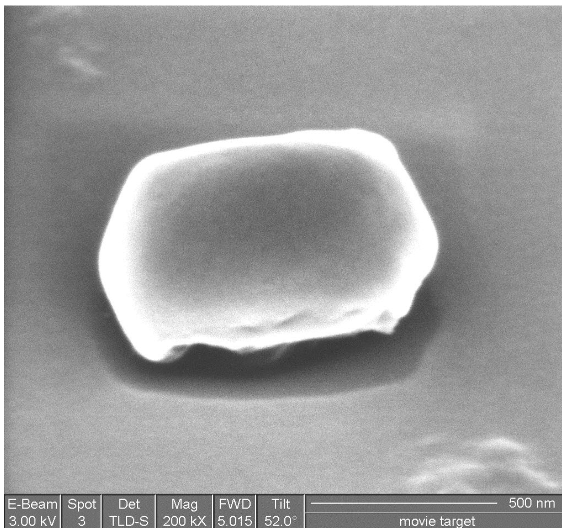


Figure 5. High magnification SEM image of a Pt coated *Bacillus* spore.

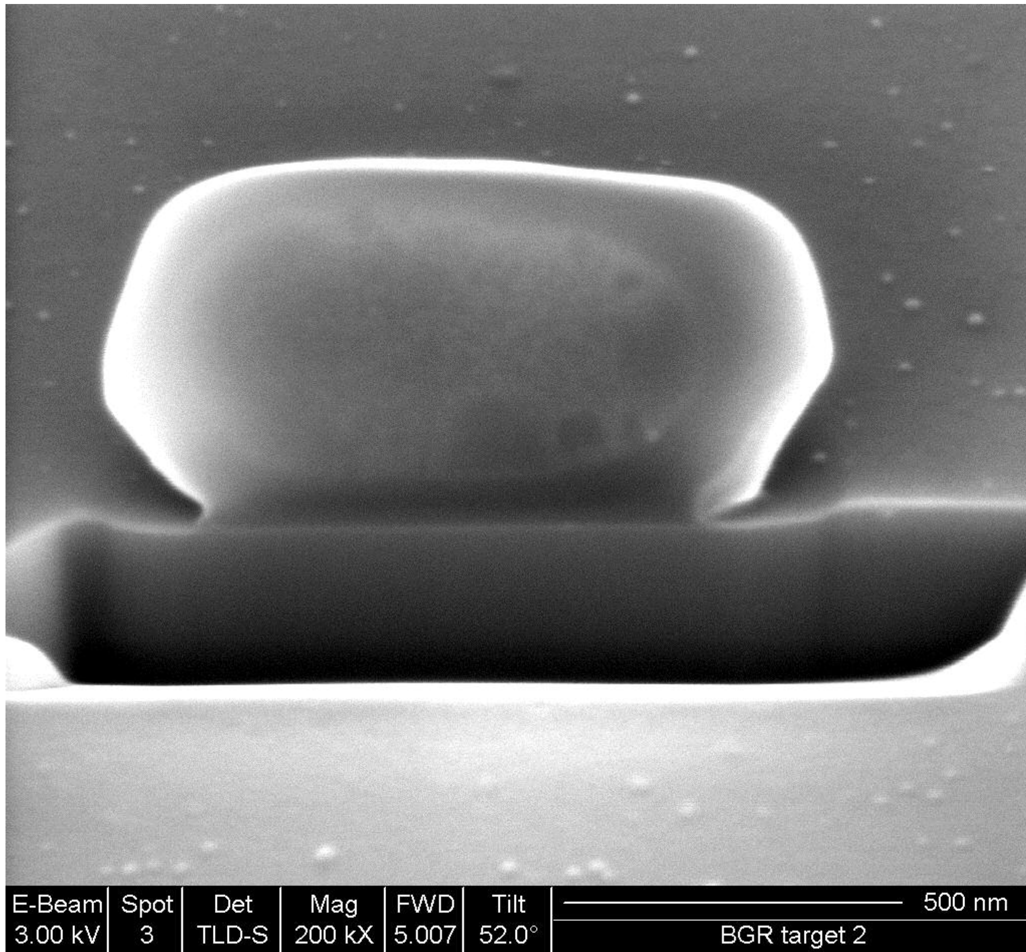


Figure 6. High magnification cross-section SEM image of the internal structure of a single spore.

Figure 6 shows a cross section SEM image of the internal structure of the *Bacillus* spore after ion beam sectioning. An outer coating layer, the combination of the Pt coating and the spore coat, having a thickness of ≈150 nm can be seen. From this image the uniformity of the coating layer can also be observed and measured. In the central region of the spore internal structure can be observed. The overall microstructure is very low in contrast in the SEM mode. Minimal image enhancement has been performed on this image in order to increase the image contrast.

The sectioned spore was then rotated 90 degrees such that the cross-sectioned surface was positioned normal to the ion beam. The cross-section surface was then ion

etched for approximately 5 seconds using the same ion beam conditions as the sectioning process. Figure 7 shows that the contrast of the internal structure has improved.The increased contrast allows a more accurate estimation of the sizes of the spore components. The total length of the spore seen in Fig 7 is measured to be approximately 1062 nm, and its diameter is approximately 500 nm, including the Pt coating. The outer layer (which appears homogeneous in the image) is a concatenation of the Pt coating, outer spore coat, inner spore coat and spore cortex, and is approx. 156 nm thick. The spore core appears in the image as the heterogenous center of the spore and is found to be approx. 750 nm in length. Continued etching increased image contrast but the image was no longer interpretable, most likely as a result of ion damage and differential sputtering effects.

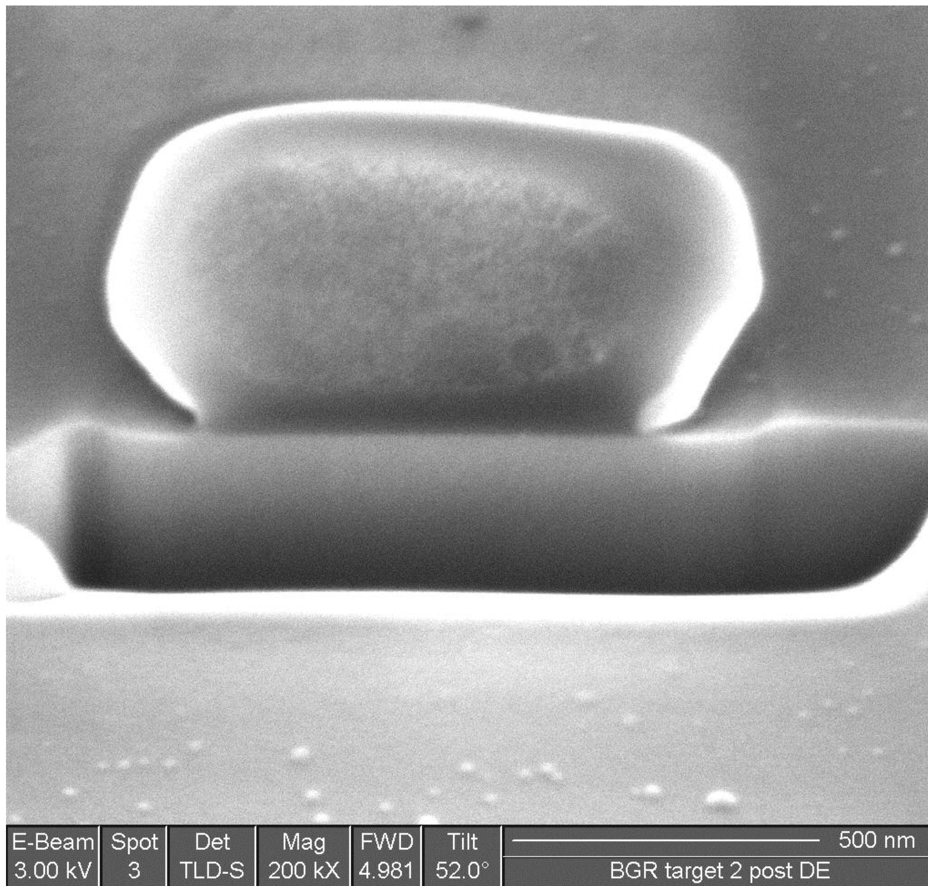
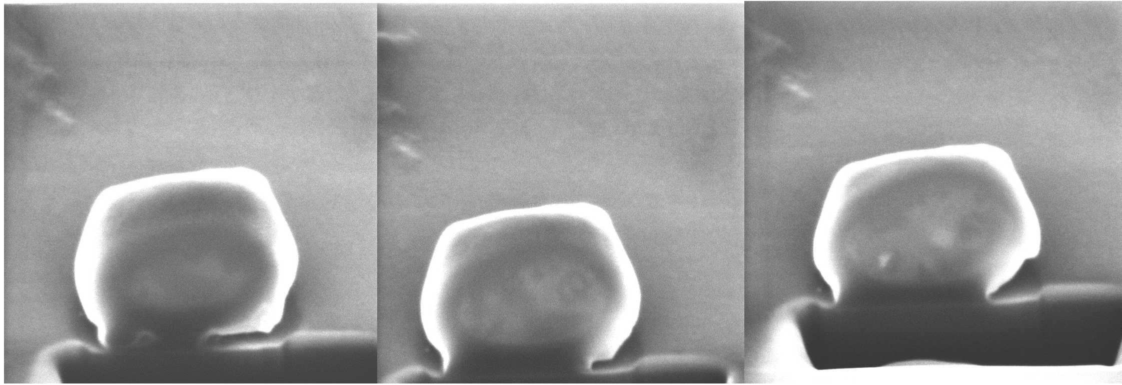


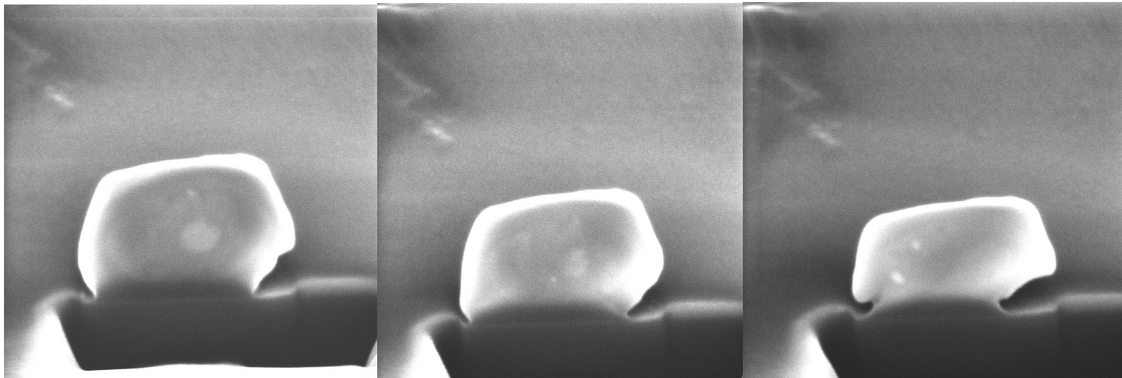
Figure 7. SEM image of ion etched cross-sectioned surface of a *Bacillus* spore.

Next, a serial sectioning process called "slice and view"™ was performed on yet another isolated spore. The slice thickness was approximately 0.08µm with the same

previous ion beam conditions. The time for each slice was ≈10 seconds. Figure 8 shows several of the sections.



Figures 8a. 8b. 8c.



8d. 8e. 8f.

Figure 8 a-f. A "slice and view" serial section. Images a-f are 6 successive SEM images at approximately 0.08μm steps.

Mark: is the light "halo" around the outside of the spore due to the Pt coat?

From this serial sectioning process we can easily observe differences in internal structure as a function of position within the spore as well as how the spore coating uniformity changes through out the spore. Fig 8a shows the initial slice removed from the outside of the spore; the dark ring is the spore coat/cortex, while the lighter inner region is the spore core. Figs 8b-8d show increasing amounts of detail as the slices go through the center of the spore. As expected, the spore coat is larger in diameter near the center as well. As the center of the spore is systematically removed, Figs 8e-8f show primarily the

remaining spore coat. The entire sequence of automated slicing and SEM imaging of each slice took ≈ 2 minutes.

Three attempts towards the demonstration of nano-machining of spores were performed. These include drilling a small hole, filling the hole and making an attachment to the spore. Figure 9 is a high magnification SEM image a single spore with a 0.10μm

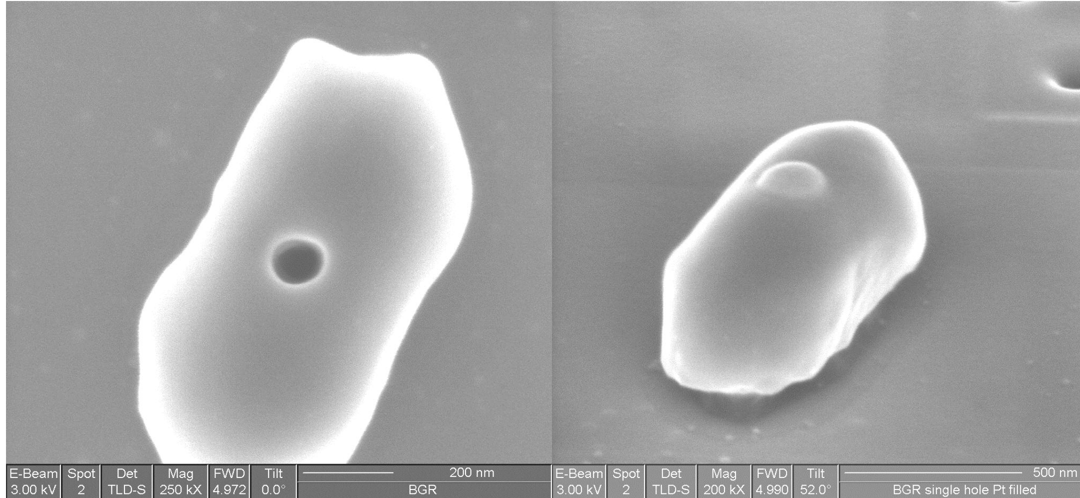


Figure 9. SEM image of a hole drilled into the top of the spore.

Figure 10. SEM image of a Pt plug filling the hole.

diameter hole drilled through its center. Figure 10 is the same spore seen in Figure 9 after filling the hole with Pt metal via ion beam deposition in a Pt gas atmosphere. Figure 11 is an SEM image of another single spore where we have deposited Pt via ion beam deposition in two steps in order to hold the spore in place and/or to make a conductive contact with the spore. The ion optical conditions for these micro-machining processes were similar to the previous cross-sectioning and deposition parameters.

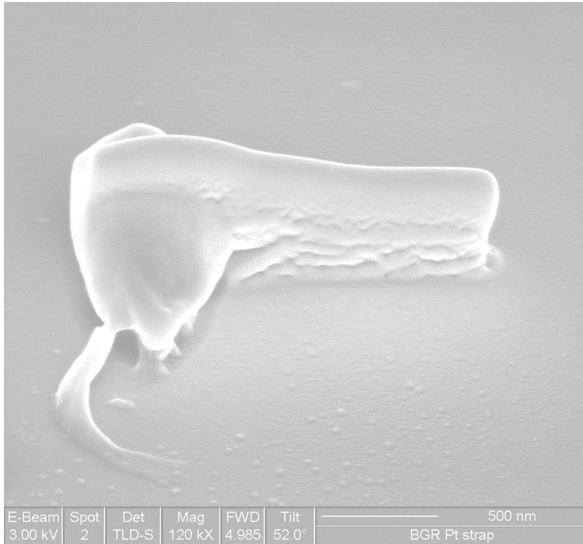


Figure 11. An SEM image of a spore having a Pt metal "strap" attached to the specimen.

Discussion

In a dual beam FIB equipped with a field emission electron source there appears to be minimal compromise in secondary electron imaging of spores compared with a conventional high resolution SEM for the magnifications employed here. The instrument that we used is capable of low voltage imaging in the SEM mode. We could have experimented with voltages as low as 500 eV to see if coating was necessary, if time had permitted. Lower voltages are also advantageous because they would provide more structural details of the *Bacillus* spores. The one unique aspect of a dual beam system regarding coating of samples to reduce the effects of charging is the ability to selectively pick the area and/or features to coat instead of having to coat the entire sample and mounting surface. Additionally, one can determine the minimum amount of coating necessary by using an iterative process of minimal coating and imaging *in situ*. Another option to be explored is to use the electron beam for deposition coating. This is a slower process but would not result in etching the sample during the initial stage of deposition as with the ion beam deposition process. The coating of these spores is an important issue since atomic force microscopy has detected nanometer size features on the surface of the spores and coated SEM spore samples has yet to image these features. The coating most

likely obscures the imaging of these small features. For the FIB sectioning process the coating was absolutely needed to eliminate charging during ion sectioning. If charging occurs during ion milling, the ion beam will move and this will in turn effect the precision of the cutting.

From our results ion beam sectioning or machining of spores at the nanometer scale appears to be very possible and in fact straight-forward. Using the electron micrographs of *Bacillus* spores in Fig. 1 of the review by Driks (1) as a reference point, we find that the sizes of the spore structures we observed compare well. Although the actual spore we measured is somewhat smaller in length than that in the Driks paper (1062 nm vs 1482 nm), the relative sizes of the structures in the two samples are similar. The internal structure, particularly the heterogeneity of the spore core, is also consistent with others. It is commonly thought that the core of a spore contains a gel in which cross-linking between macromolecules occurs through stable but reversible bonds so as to form a polymeric matrix (2). Although we cannot identify the particular molecules with this technique, our results certainly reveal a matrix-type material in the spore core. The ion etching rate is extremely high. This allows use of the smallest ion beam aperture size and lowest current, thus leading to very high resolution sectioning with few or no obvious induced artifacts from heating. Additionally, with a thin layer of Pt coating and a small diameter ion beam, there does not appear to be any rounding off of the sample and the cut surfaces are very smooth. It is difficult to assess ion damage without performing a TEM cross-section of the ion-sectioned surface. In other materials ion damage is on the order of 10 nm. Since it appears that there is reasonable secondary electron contrast and the typical escape depth of the imaging secondary electron is on the order of several nanometers, it appears that ion damage is minimal. Ion etching of the specimen surface at a 90-degree angle initially gives greater secondary contrast. The topography that evolves with etching to give increased contrast rapidly develops into artifact from redeposition and differential sputtering. This should be considered when trying to perform any type of analytical ion beam analysis on these materials. Finally, the ion beam sectioning could also prove important for the site-specific sectioning or harvesting of samples for surface analysis such as nano- and ToF-SIMS and EDS in the SEM and TEM techniques.

The ion beam micro-machining of the spores proved to be quite straight forward, similar to other established FIB processes on metal and semiconductors. Again, because of the small ion beam diameters and low currents, a small hole was easily drilled into the specimen. Combined with the Pt deposition to plug and connect to a spore, one could perform measurements such as electrical resistivity.

Conclusions

We have reported on the first use of dual beam techniques on *Bacillus* spore material at LLNL. The process of imaging and sectioning has been shown to be precisely controlled with little damage to the sample. LLNL has many analytical techniques capable of analyzing a single spore. However, while single spore or cell measurements are essential to learning about the variability in a sample, it is very important that the samples are prepared consistently to ensure that any measured differences are real and not artifacts of the preparation process. The ability to consistently and precisely cut samples (spores) will greatly enhance the spatial resolution of other analysis techniques such as ToF-SIM and nano-SIMS. These analytical techniques have a spot size on the order of 50-150 nm to perform very high resolution measurements, but their capability is much enhanced by having a precision sample. Because the FIB can "slice and view"™ samples, these analytical techniques will be able to obtain a “depth profile” in a controlled and consistent manner. This allows for correlations to be made between the results with the confidence that the same spore area was being analyzed by both.

Furthermore, the ability to micro-manipulate and micro-machine spores opens the door to many unique experiments heretofore unavailable to the researcher. As mentioned, by using gas deposition to create electrodes to/from the spore, electrical conductivity and stimulation experiments could be performed to determine the effect of electrical currents on biological processes such as spore germination. In combination with an SEM or TEM, imaging of the processes could take place in nearly real-time.

In summary, the FIB combines high resolution imaging with sample sectioning and micro-machining to generate a unique tool for biological sample preparation and analysis.

References

1. Driks, A. (1999) *Microbiol. Mol. Biol. Rev.* **63**, 1-20.

2. Black, SH and Gerhardt, P. (1962) *J. Bacteriol.* **83**, 960-967.