Rapid Processing of *Bacillus subtilis* for 3D Analysis by FIB-SEM.

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Anthrax is an acute, often lethal disease caused by the bacterium *Bacillus anthracis*. *B. anthracis* forms endospores which constitute its primary mode of transmission. Formation of these endospores through a process called sporulation is highly regulated both spatially and temporally in *B. subtilis*, a model organism amenable to laboratory studies. The high-resolution 3D characterization of endospore structure within the bacterium can be achieved using a recently developed microscopic technique called focused ion beam scanning electron microscopy (FIB-SEM) [1]. However, sample preparation is long and involved, typically taking 3-4 days. Previous studies have shown that microwave processing of both bacterial cultures [2] and bacterial endospores [3] for standard transmission electron microscopy (TEM) decreases processing time without a loss in ultrastructural preservation. Here we adapt microwave sample preparation protocols for 3D electron microscopy by FIB-SEM.

Cultures of *B. subtilis* in various stages of sporulation were pelleted and fixed in 2% glutaraldehyde in 0.1M sodium cacodylate buffer with half of the samples exposed to fixative for 30 min at room temperature and half of the samples exposed to fixative for a total of 3 min in the microwave. All samples were then rinsed in buffer and processed in the microwave through the following steps: 1% osmium tetroxide, 10 min; 1% uranyl acetate, 2 min; dehydration in an ethanol series, 4 min; and infiltration with hard Epon resin, 15 min. The two sets of samples were then polymerized either in the microwave for 1.25 hr or in a 60°C oven for 2 d. Initial analysis of samples was then performed in the TEM. Additional analysis was done in the FIB-SEM.

Use of microwave processing decreased the amount of time required to prepare samples for FIB-SEM analysis from 4 days to as little as 4 hours. Ultrastructural preservation was comparable between microwave and conventional fixation procedures. The cell wall, plasma membrane, and endospore coat layers were more distinct in the conventionally-fixed, oven-polymerized samples, while certain features within the cytoplasm and the endospore core stained better with microwave polymerization (Figure 1). TEM micrographs also revealed the occasional presence of holes within the bacterial cells; these were observed in all samples with comparable frequencies, suggesting the artifacts were unrelated to microwave fixation. These data show the compatibility of microwave processing for 3D analysis by FIB-SEM and are a first step toward rapid, routine, high-throughput, high-resolution 3D EM analysis of biological samples (Figure 2) [4].

References:

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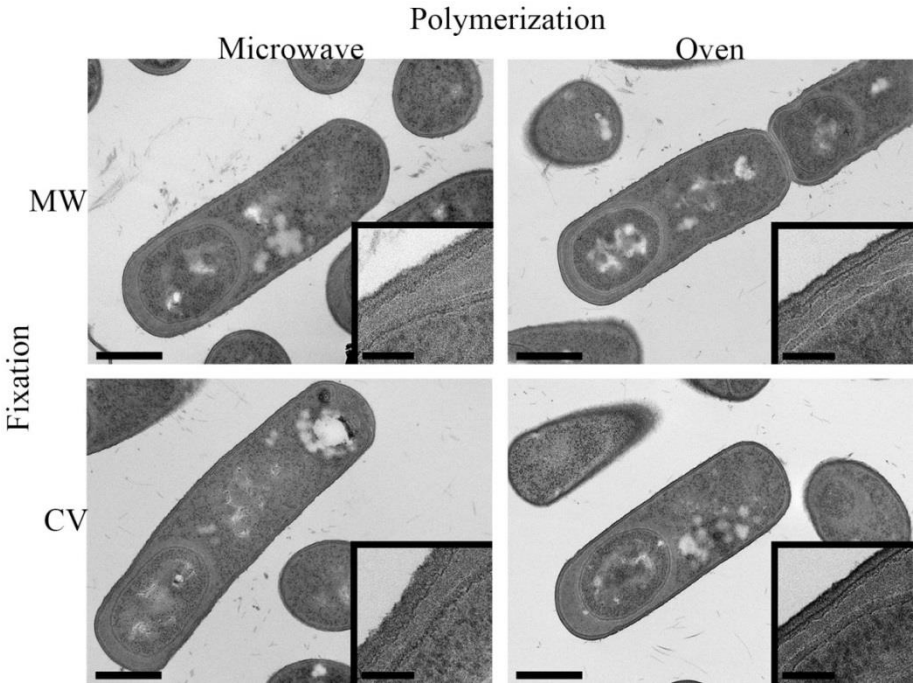

Figure 1. *Bacillus subtilis* pellets were fixed in the microwave (MW) (top row) or conventionally on the benchtop (CV) (bottom row), then processed through the microwave. Pellets from each fixation treatment were polymerized in either the microwave (left column) or in a 60°C oven (right column). Cell walls and endospores are more distinct in the CV-fixed and oven-polymerized samples. Bar = 500 nm. Insets show a magnified region including the cell wall and endospore coat layers. Inset bar = 100 nm.

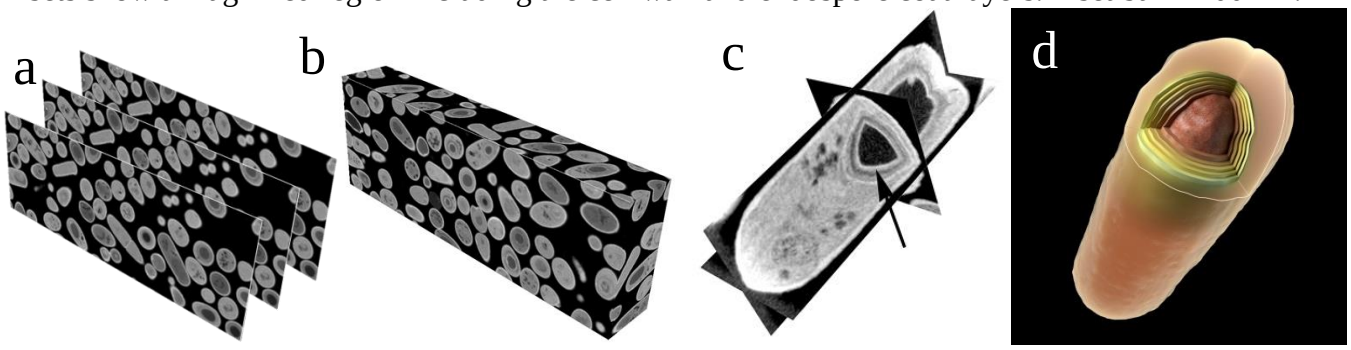

Figure 2. In FIB-SEM, a resin-embedded *B. subtilis* pellet was subjected to a protocol of iterative milling or "slicing" by the FIB and imaging by the SEM to yield a stack of 2D images (a), which is then computationally converted to a 3D image volume (b). The bacteria are thus imaged at nanoscale resolutions in all 3 planes (c), allowing the visualization of features such as the layers surrounding the endospore core (arrows in c, artist's depiction in d).