Application Note

Microbiology

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Quantitative Image Analysis of Bacilli Viability in Agar Mounted Soil Samples

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Abstract

Bacterial cell viability is a useful measure to indicate microbiota stress, status of pathogenic strains in environmental samples, or efficacy of induced chemical treatments for example. A traditional method for determining viability uses a pour plate method, where a direct plate count of microbial culture growth prepared from a sample colony suspension is performed manually.[1] This procedure can vary from one to several days or more depending on the strain. Fluorescent staining provides a rapid way to determine bacterial viability with or without culture, and as shown here is conducive to quantitative analysis using automated microscopy. In addition to obtaining a relative percent of viable cells, by differentiating stained objects in a sample using morphology metrics, spore content in relation to vegetative cells can be calculated as an additional indication of viability of the sample bacteria.

Introduction

Healthy microbiota depend on a variety of bacterial strains that each play a unique role to sustain homeostasis. These organisms populate environments as diverse as air, hypersaline ponds, volcanic soils, and mammalian, avian, and insect digestive tracts, and are subject to numerous organic and induced changes in their hosts including nutrient availability, dehydration, pH, temperature, and chemical toxins. Due to their resilience, prevalence, adaptive mechanisms, and role as both beneficial and pathogenic organisms, bacteria are the target of multiple fields of research covering a broad range of applications from development of drug compounds and delivery systems to manufactured food products.

The primary adaptive success of any cell is the ability to survive and reproduce. Some bacteria, for example *bacillus subtilis*, a bacteria common in surface soil, possess more than one reproductive pathway depending on microbiota conditions. *B. subtilis* can multiply both by vegetative medial cell division during favorable conditions, and by sporulation when induced by environmental stress, notably nutrient deprivation (Figure 1). Bacterial spores contain all genetic material of the strain, can be resistant to severe conditions such as radiation, desiccation, and hypoxia, and dormant spores millions of years old have been germinated to vegetative growth stage.[2,3] In this application note, a method for imaging and analyzing viability of bacilli isolated from soil samples is described.

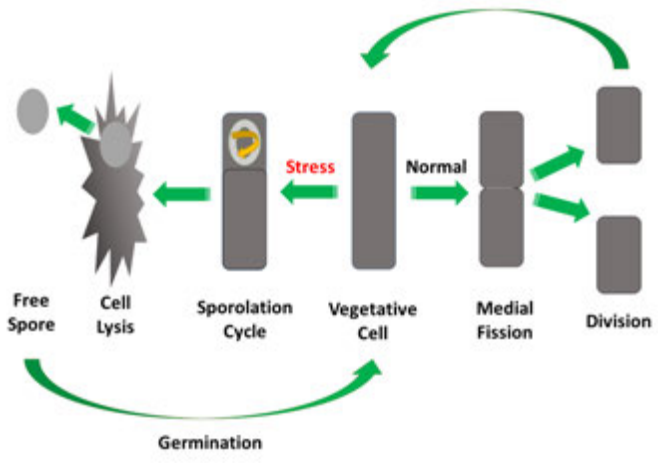

Figure 1. Overview of endospore-producing bacteria cell cycle.

Materials and methods

Methods

Staining assay

Bacillus enriched and isolated from a soil sample, stained with Syto 9 and propidium iodide (e.g. Thermo Fisher Scientific LIVE/DEAD *Bac*Light Bacterial Viability Kit, for microscopy, part number L7007) and mounted stabilized either as an agar smear or on an agarose-coated microscopy slide, was prepared by a university researcher and submitted for image analysis. There was no information on the origin of the sample, or whether any induced environmental stress, inactivation procedure, or chemical treatment had been applied. It is assumed bacilli were enriched and isolated from soil using a common selection technique such as a spread-plate method.

Imaging assay

The slide was placed into an Agilent BioTek slide adapter (part number 1220548) coverslip down and loaded onto the Agilent BioTek Lionheart LX automated microscope (per request) controlled by an Agilent BioTek Gen5 microplate reader and imager software. Slide scanning was performed in brightfield using a 4x objective (part number 1220519) to find regions of interest (Figure 3). A 60x objective with a correction collar setting of 0.17 (part number 1220545) was used for additional verification of bacilli objects (Figure 2). A 20x objective (part number 1220517) with a correction collar setting of 0.17 was used to capture images for analysis in brightfield and fluorescence with a combined GFP (Syto 9) filter cube (part number 1225101) with 465 nm LED cube (part number 1225001), and a combined PI (propidium iodide) filter cube (part number 1225111) with 523 nm LED cube (part number 1225003). Image preprocessing was applied to GFP and PI channels using a rolling ball size of 2 µm with fine results and a smoothing factor of 2. Background gradation and unresolved images in brightfield indicated not all objects may lie in a single focal plane (Figure 4). A z-stack was therefore defined by finding a minimum and maximum focal height scanned at 0.5 µm focal height adjustments in GFP and PI. This focal scanning was used to determine a final z-stack definition of 9 images covering a range of 48 µm at 6 µm focal height intervals. Z-stacks on preprocessed GFP and PI images 1 through 7 (origin focal height +0 to +36 µm) were combined into a final z-projection using a method of Maximum. To obtain a total cell count a primary mask was defined on GFP stained objects with a size from 0.8 to 8 µm, a detection threshold of 3,421, and the options to exclude edge objects and split touching objects chosen.

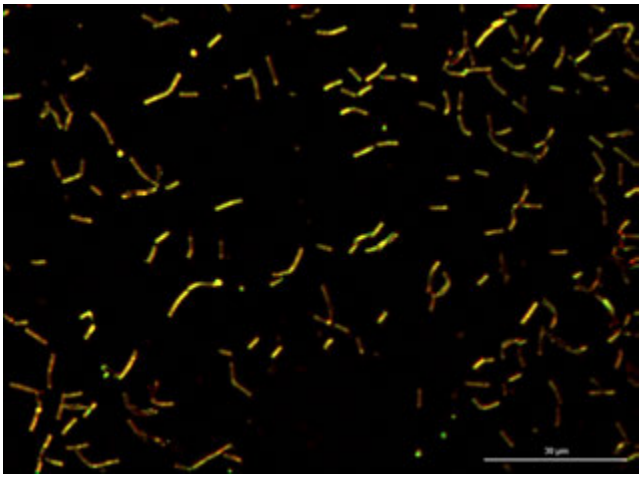

Figure 2. Merged image of soil bacilli stained with Syto 9 and propidium iodide (image captured on an Agilent BioTek Lionheart LX using a 60x dry objective). In comparison to a pour plate viability method that can take days (not shown), fluorescent staining is a rapid alternative taking only up to 2 hours depending on the staining protocol.

A secondary mask was defined on PI within a reduced perimeter area of the GFP mask (1×10^{-7}) using auto threshold to obtain an automatic object count of dually stained objects. A [PI_Pos] subpopulation was defined on objects greater than or equal to the lowest PI mean signal intensity from the secondary mask analysis of the z-projection (>8,000), with the same value applied to two individual focal heights for comparison. Spore-like objects were differentiated from vegetative and/or germinating cells using metrics of size (≤2.7), circularity (>0.3), and a custom defined aspect ratio (length/width ≤1.6). Percent spores were calculated using a scatter plot. The percent of PI positive objects in the z-projection was calculated using a scatter plot following normalization to exclude spore objects from the total count via an additional cellular analysis step.

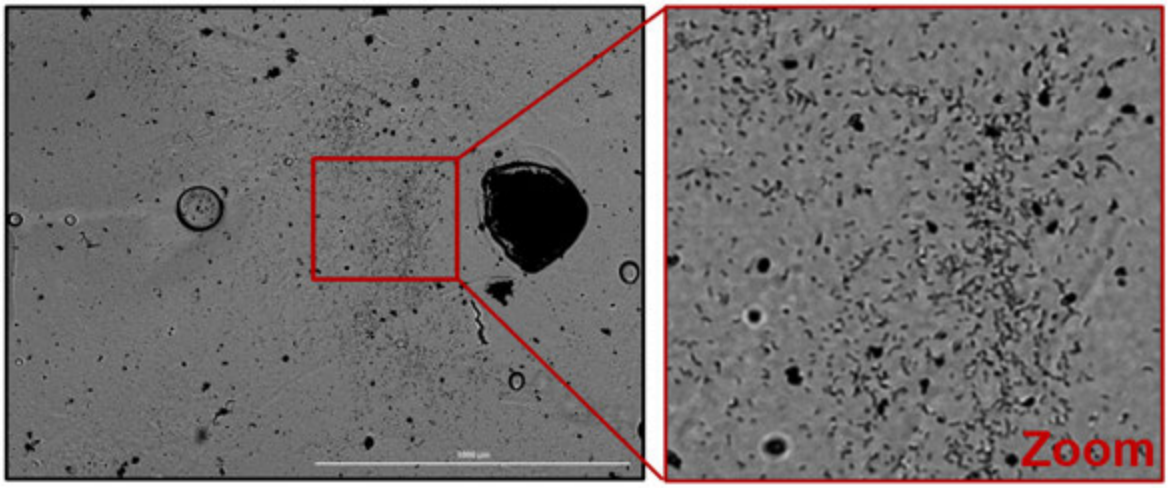

Figure 3. Slide scanning at low magnification revealed regions of interest (4x brightfield raw image).

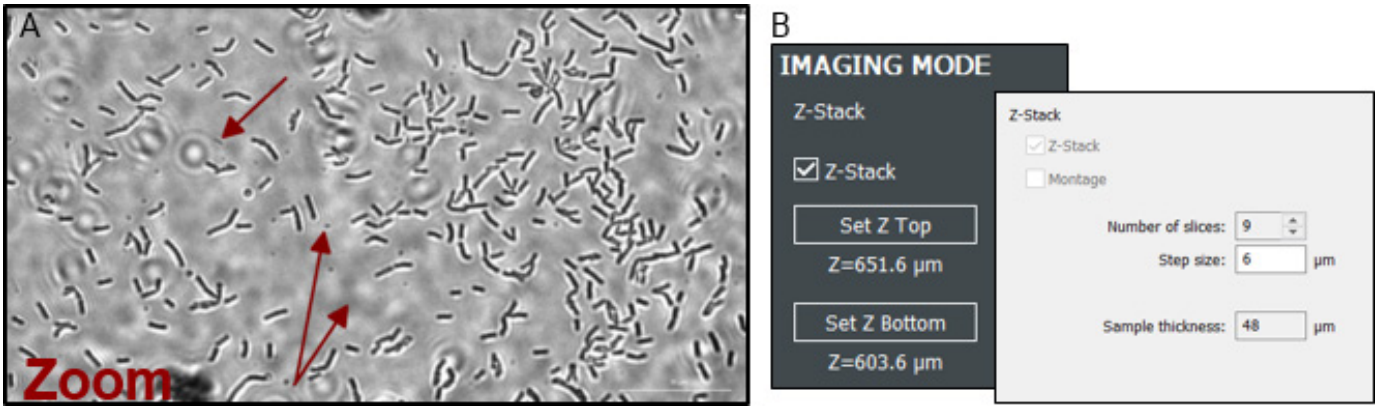

Figure 4. (A) Unresolved objects and background gradation (arrows) indicated additional targets may exist that lay outside a single focal plane (20x brightfield raw image). (B) Z-stacking was used to automatically capture multiple images at different focal heights.

Results and discussion

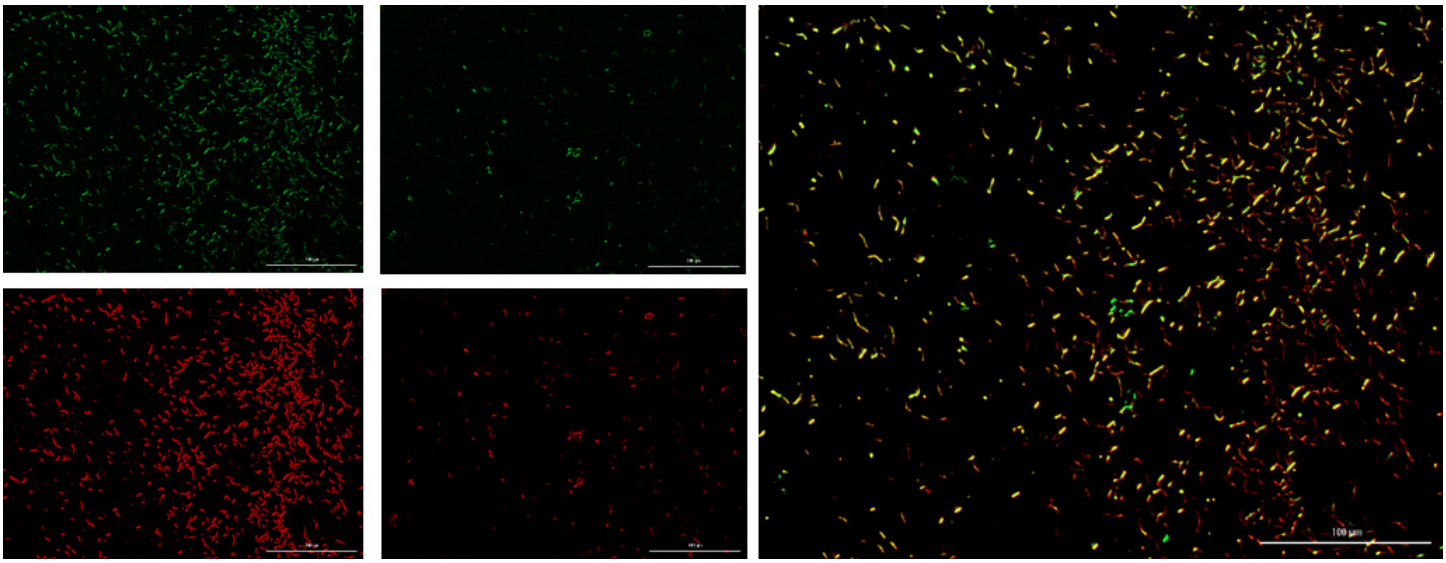

Figure 5. A z-projection was defined on a custom focal range of GFP and PI images taken from z-stack imaging (20x, two focal heights for GFP and PI, merged z-projection).

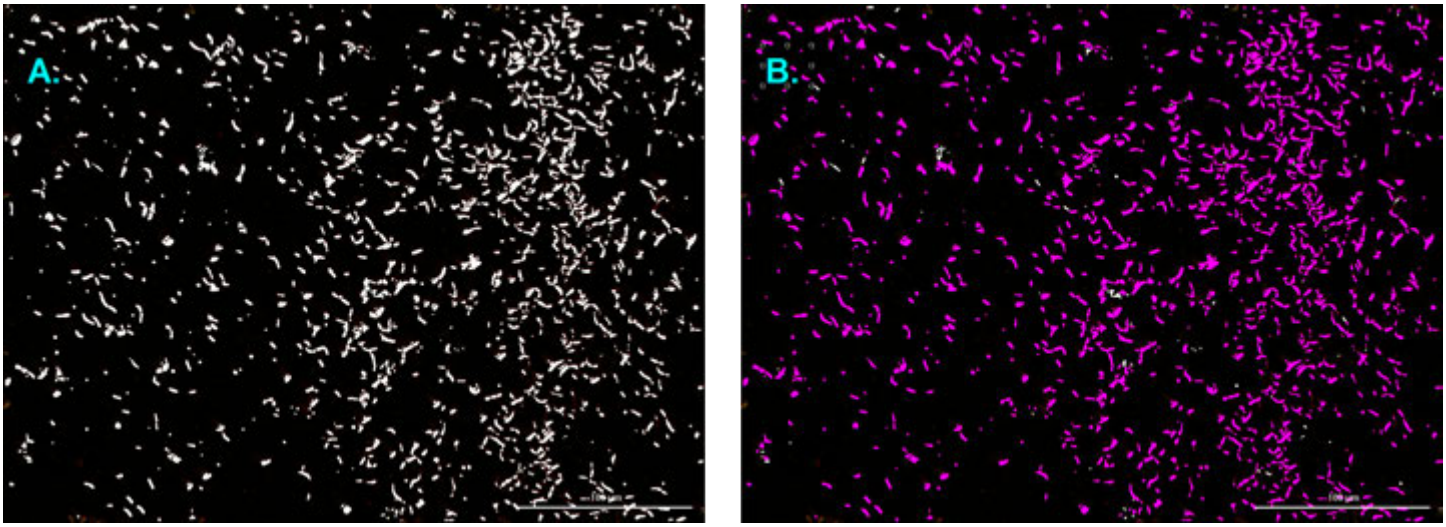

Figure 6. (A) A primary mask was defined on the z-projection for objects meeting size and detection thresholds in GFP to obtain total cell counts (white); (B) a subpopulation analysis using results of a secondary mask on PI (purple) was used to calculate percent PI positive (nonviable) cells.

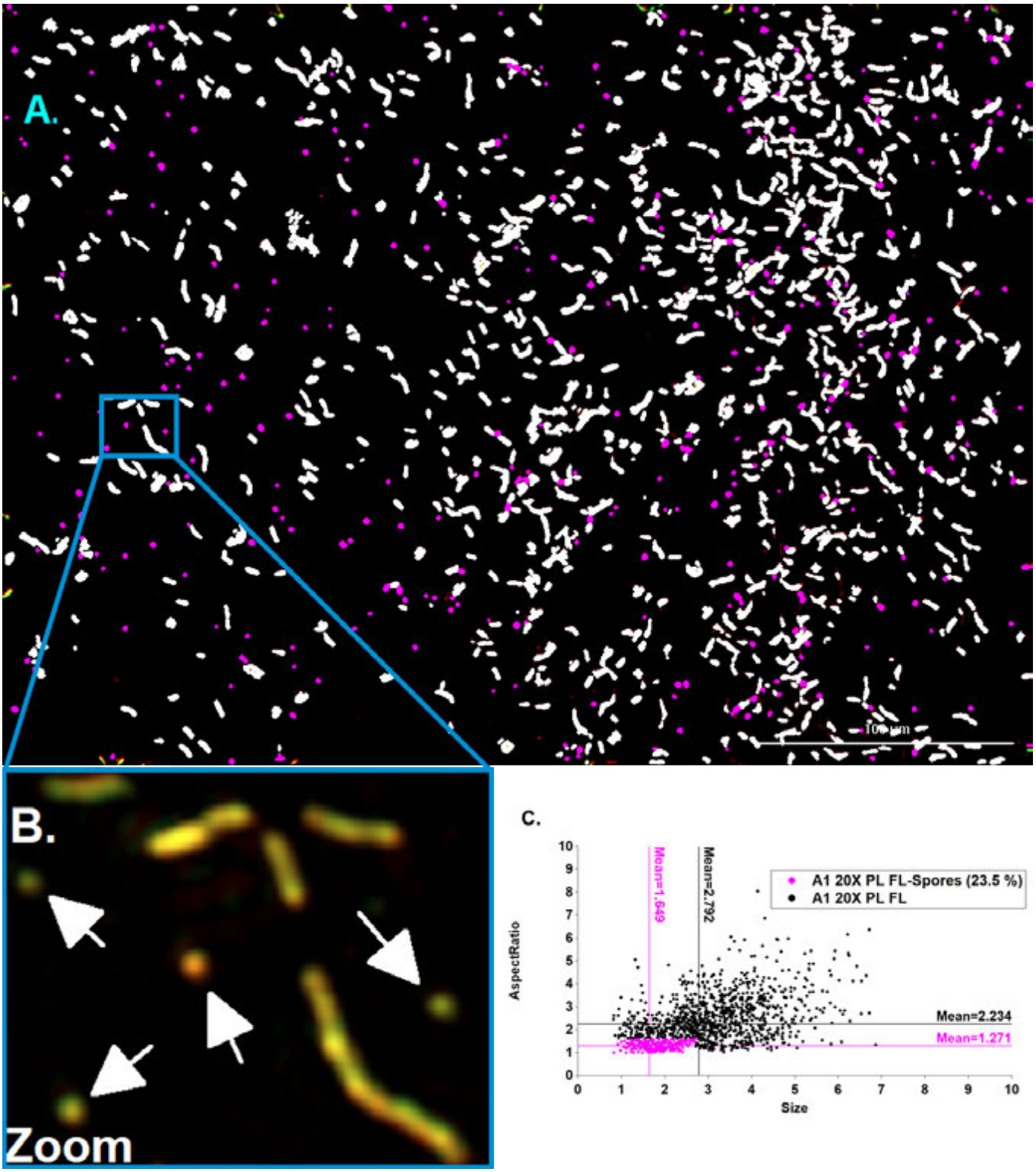

Figure 7. (A) A subpopulation analysis was done to differentiate spore-like objects (purple); (B) objects defined as spores (white arrows) are shown among vegetative cells (unmasked, 20x image zoom); (C) a scatter plot illustrates spore characteristics and calculates percent spores compared to the total population.

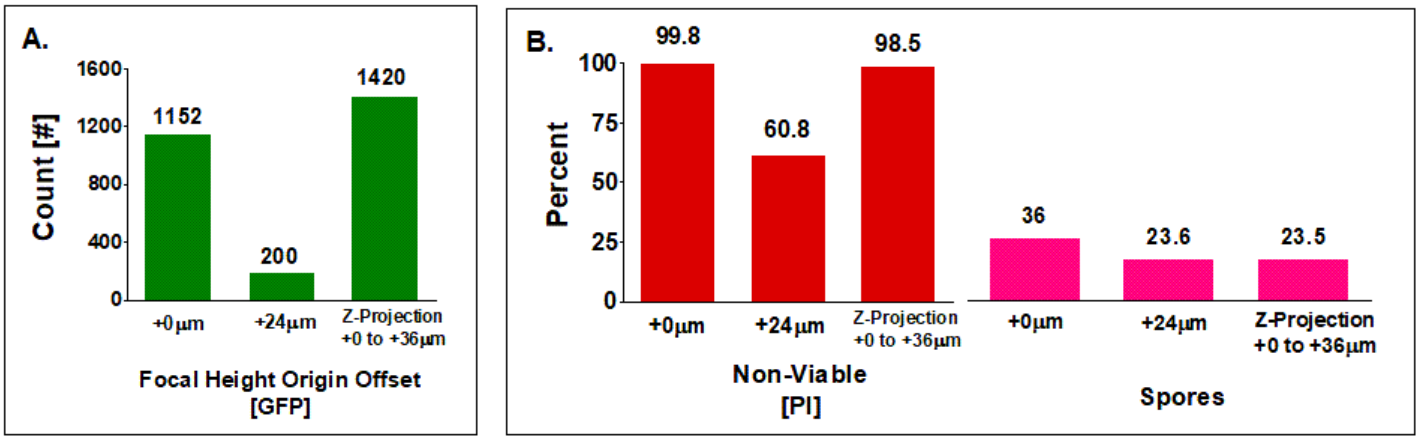

Figure 8. (A) The z-projected image resulted in a higher count compared to individual focal height images; (B) a relative viability profile of the sample is shown by both total objects that are PI positive and a percent of spore-like objects. Uptake and/or other binding of PI by spores was observed. It has been suggested that PI staining of spores may not be specific for viability,[4,5] therefore the total count was normalized on the spore count before calculating percent nonviable cells.

Conclusion

A rapid, reproducible method to analyze and quantitate sample viability of fluorescently stained soil bacilli mounted in agar on a microscope slide was described using an Agilent BioTek Lionheart LX automated microscope and the Agilent BioTek Gen5 multimode reader and imager software. A 4x objective was found adequate to scan slides for regions of interest, and a 20x objective was suitable for counting and analysis. Higher magnification is available for additional imaging options if desired. A z-stacking and z-projection feature was implemented to capture objects over a range of focal heights and was found to increase microbial counts analyzed here from (23 to 610%) over counts from single image planes. A secondary mask and subpopulation analysis on PI resulted in z-projected viability counts 37.7% higher and 1.3% lower than representative single images. Subpopulation analysis was also used to differentiate a population of spore-like objects from the total object count. A scatter plot was used to calculate the percent and illustrate the characteristics of these objects. The percent of spores detected ranged from a high of 36% in the lowest offset image to 23.5% in the z-projection. Although this imaging assay could be useful to improve accuracy of total counts in a sample, viability analysis was less sensitive at one focal height, suggesting that, as expected, resolution of smaller objects is more susceptible to focal height offset. Further, although the same detection threshold was applied to all data shown, in the normalized cellular analysis the detection threshold for GFP staining was lower for 2.7 µm ≤ isolated objects ≤8 µm in the z-projection (data not shown). Resolution and analysis may therefore be enhanced for both total count and viability by decreasing the focal height offset of the z-stack to less than 6 µm. Moreover, size and signal thresholds are interdependent; however, to preserve data integrity, the same settings were applied to each analysis step. The Lionheart LX is equipped with four fluorescent channels and a variety of acquisition modes that would accommodate an increase to the analysis content shown here either by supplemental staining or capturing a montage of multiple image areas. The Agilent BioTek Lionheart FX, Cytation 5, or Cytation 1 are also compatible with this imaging assay and include a phase contrast option that could further complement vegetative and endospore imaging.

References

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