A Rapid Single Spore Enumeration Assay

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Abstract[1,2]—The Rapid Single Spore Enumeration Assay (RapidSSEA) is being developed for the Mars Technology Program to aid planetary protection personnel in their efforts to validate bioburden reduction on spacecraft surfaces prior to launch. RapidSSEA is based on imaging and counting individual bacterial spores in a microscope field-of-view, where the contrast is generated by a highly luminescent complex that forms when dipicolinic acid (DPA) is released from spores during germination and binds to terbium (Tb^{3+}) ions in the surrounding medium. Under pulsed UV excitation, the long-lived ($\tau \sim 1$ ms) DPA-triggered Tb luminescence is imaged by a lifetime-gated camera, essentially eliminating fluorescent interferents from the image, which enables counting of bacterial spores swabbed from environmental samples.

In practice, the RapidSSEA analytical procedure entails swabbing bacterial spores from the surface to be analyzed with cotton applicators, and resuspending them from the applicators into water, which up to this point is identical to the NASA standard assay. Filtration of the resuspended sample concentrates and immobilizes the bacterial spores onto a membrane filter. Spores are then transferred onto Tb^{3+}- and L-alanine-doped agarose by streaking the membrane filter across the agarose surface. Finally, individual spores, or more specifically the resultant Tb-DPA luminescence halos surrounding the germinated spore bodies, are imaged and counted using a lifetime-gated microscope.

Here we report results from the first side-by-side comparison of RapidSSEA and the NASA standard assay. Both assays were used to assess the spore population on 2.25"×2.25" (5.7 cm × 5.7 cm or 32.5 cm^2) stainless steel coupons inoculated with 1.3×10^5, 5.0×10^4 and 7.0×10^3 *Bacillus atrophaeus* spores. Using RapidSSEA, 38.0% ± 5.8% of the total inoculated spore population was recovered and counted within 30 minutes. The NASA standard assay recovered and counted 7.4% ± 2.7% of the inoculated spores, but required 3 days before results became available. In addition, we report a concentration dependence experiment, which demonstrated a 5000 spores/coupon detection limit for RapidSSEA due to background luminescence, contamination and difficulties in counting. Finally, we discuss future work that is envisioned to improve the detection limit to less than 10 spores/coupon.

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1. INTRODUCTION

Bacterial spores are dormant structures formed during the resting stage in the life cycle of genera such as *Bacillus* and *Clostridium*. They are formed within vegetative cells during sporulation, which is frequently triggered in response to adverse changes in the environment. The DNA of a spore is protected from the environment by a surrounding spore coat comprised of calcium ions, DPA and protein layers. It is believed that DPA contributes to heat and UV resistance [1-3]. With no detectable metabolism, bacterial spores can persist in environmental extremes, such as freezing [4,5], boiling [6], pressure [7,8] and desiccation [9], and remain viable for many years [10]. More controversial reports claim that spores entombed in a bee trapped in amber 25-40 million years ago have been revived [11], and an even more spectacular claim details the recovery of spores from 250-million-year-old halite crystals [12]. The extreme resistance of bacterial spores has been taken advantage of to check the performance of autoclaves [10, 13, 14]. Once favorable conditions return, bacterial spores germinate to produce vegetative cells [15]. Bacterial spores have also been invoked as the most likely interplanetary travelers that might survive such a journey [16], which underscores their importance as a metric for the validation of spacecraft bioburden reduction.

The NASA Procedures and Guidelines document NPG 8020.12b [17] describes planetary protection provisions for robotic extraterrestrial missions. Among the required provisions for category III-V missions is a terminal microbiological assay that determines spacecraft bioburden levels prior to launch. For example, Class IVa planetary missions, comprising landers and probes without life-detection experiments, must meet a bioburden limit of 3×10^5 spores/vehicle (and less than 300 spores/m^2). Missions with life-detection experiments must undergo additional procedures to ensure that the total bioload does not exceed 30 spores [18].

Currently, bioburden levels are determined using the NASA standard assay, with which bacterial spores are quantified in terms of colony forming units (CFU) that become visible on growth plates after incubation. There are several limitations for the NASA standard assay. First, this process requires 3 days to complete. Thus, when plate counting is employed as the terminal microbiological assay, the spacecraft must be protected from recontamination. Second, a large number of bacterial spores can aggregate on individual particulates giving rise to a single CFU, and is thus a large underestimation of the bioburden. Third, colony-counting methods only account for cultivable spore-forming species, which constitute less than 1% in soil samples [19-21]; a significant component of spacecraft contamination is known to come from soil [18].

The most significant advantage of RapidSSEA is that it takes less than 30 minutes to enumerate spores on a surface. In addition, RapidSSEA does not require full colony formation to obtain a spore count, but only stage I germination during which DPA is released from the spores. Thus, non-cultivable sporeformers can be counted as long as they can germinate under the experimental conditions provided. RapidSSEA thus addresses some limitations of the NASA standard assay, and is in many ways complementary in that the viable spore count is under-estimated by the NASA standard assay and over-estimated by RapidSSEA. Unfortunately, it is currently not possible to directly assay viable spore counts since viability within a given population of environmental bacterial spores is expressed under widely ranging experimental conditions such as availability of nutrients, water and temperature.

Since RapidSSEA counts a spore after germination, we report spore counts in terms of germinating spore units (GSU) rather than colony forming units (CFU). GSU can be determined faster than CFU because onset of germination requires only several minutes [22-25], whereas a colony takes days to form.

The advantages of RapidSSEA are gained because it is based on a molecular approach. From the perspective of the detection scheme, the bacterial spore is, in essence, a 1-μm diameter bag filled to very high concentrations with a unique chemical marker, dipicolinic acid (DPA). DPA is found only in the core of bacterial spores [15], and can be released from the core into the surrounding volume by inducing germination (e.g., with L-alanine [26]) or physical lysing (e.g., autoclaving, microwaving [27]).

When DPA is released from the spore into a surrounding medium containing terbium ions (Tb^{3+}), highly selective DPA-to-Tb^{3+} binding triggers bright green luminescence when viewed under UV light [28-31]. The highly luminescent Tb-DPA complex surrounding the germinated spore bodies can be imaged and counted using a lifetime-gated camera. Lifetime gating is enabled due to the long luminescent lifetime ($\tau \sim 1$ ms) [32,33] of Tb^{3+}, and effectively removes all background fluorescence (i.e., interferent fluorophores with nanosecond lifetimes), thus rendering the background intensity for imaging silent. Elimination of this background enables a striking increase in image contrast and sensitivity for detection.

Here we report the results of (1) the first side-by-side comparison between RapidSSEA and the NASA standard assay for 2.25"×2.25" stainless steel coupons inoculated with 1.3×10^5, 5.0×10^4 and 7.0×10^3 *Bacillus atrophaeus* spores, and (2) a RapidSSEA concentration dependence experiment ranging from 10^5 - 10 GSU/coupon. We will also discuss future work that is envisioned to improve the detection limit to less than 10 spores/coupon.

2. EXPERIMENTAL SECTION

Materials

Chemicals—Terbium (III) chloride hexahydrate, 99.999%, and L-alanine were purchased from Sigma (St. Louis, MO) and were used as received. Ultrapure™ agarose (> 90% agar) was purchased from Invitrogen (Carlbad, CA). Tryptic soy agar (TSA) was obtained from Becton, Dickinson and Company (Sparks, MD). All solutions were prepared in 18.2 MΩ-cm deionized water.

Microbiological samples—Endospore suspensions of *Bacillus atrophaeus* (ATCC #9372), formerly known as *Bacillus subtilis* var. *niger* [34], were purchased from Raven Biological Laboratories, Inc. (Omaha, NE) with a reported concentration of 10^9 CFU/0.1ml. Ten-fold serial dilutions were made in methanol (Burdick & Jackson, Muskegon, MI) to obtain desired spore concentrations, which were expressed in terms of total spore units (TSU) by hemocytometer counting, CFU by TSA spread plate counting and GSU by lifetime gated microscopy. Spore suspension (LOT #1092371) was maintained in 40% ethanol at 4°C before use. It was used to inoculate coupons with a concentration of 1.3×10^5 TSU/coupon, of which 53% can germinate and 35% are cultivable. Spore suspension (LOT #1092411) was maintained in water at 4°C before use. It was used to inoculate coupons with a concentration of 5.0×10^4 and 7.0×10^3 TSU/coupon, of which 65% can germinate and 24% are cultivable.

Swab-rinse materials—Stainless steel sheets (40"×80" or 100 cm × 200 cm) were procured from the Jet Propulsion Laboratory (JPL) in-house machine shop and subsequently cut into 2.25"×2.25" coupons in the machine shop at California Institute of Technology (Caltech). Coupons were cleaned and inoculated as described in the Methods section. Cotton applicators (Puritan, Guilford, ME) of length 15 cm were used to swab the coupon surfaces.

RapidSSEA materials—The membrane filtration apparatus involves a 15-ml filter funnel, fritted glass support base, aluminum clamp and 125-ml Erlenmeyer receiving flask (figure 1). Black nuclepore® track-etched membranes (0.2

μm pore size; 25 mm diameter) were purchased from Whatman (Florham Park, NJ). These Irgalan Black-dyed polycarbonate membrane filters provide a flat, smooth and low non-specific absorption surface on which microorganisms are trapped. Press-to-seal silicone isolators (9 mm diameter; 0.5 mm deep) with adhesive backs were purchased from Molecular Probes (Eugene, OR) to serve as miniature petri dishes holding the agarose substrate.

The filter funnel, receiving flask, filter support and filter base were wrapped in aluminum foil and sterilized in an autoclave (Tuttnauer 2540E, Brinkmann Instruments, Inc., Westbury, NY) before use. The filter base was unwrapped aseptically. A piece of sterile membrane filter was placed on the filter support with sterile forceps. The filter funnel was held into place with an aluminum clamp. The filter base was connected to a vacuum aspirator (B-169 Brinkmann Instruments, Inc., Westbury, NY).

Instrumentation

The lifetime gated microscopy instrument (figure 2) consists of a stereoscopic microscope, pulsed xenon flashlamp, lifetime gated imaging module, as well as other optical components.

Light source—A custom-made xenon flashlamp coupled with the lifetime gated imaging module is manufactured by Photonic Research Systems Ltd (Salford, Greater Manchester, UK). The 24-V DC xenon flashlamp provides a broadband spectrum from ultraviolet through visible to the infrared range, overlapping with the 278-nm absorption peak of terbium dipicolinate. The window material is made from quartz such that mid-UV light can pass through. The optimal flashing rate is 300 Hz. Each millisecond pulse is synchronized with the rising edge of a standard 1.5 V TTL rectangular wave trigger. Light pulses are trailed by a "tail" of up to 50 μs. A 100-μs time delay is sufficient to discriminate erbium dipicolinate, with a millisecond lifetime, against the short-lived background fluorescence and autofluorescence, usually falling within nanosecond range.

Microscope—A Nikon SMZ800 stereoscopic microscope was used in this study. It provides a large working distance between the stage and objective as well as a large field of view for counting spores. Magnification can readily be toggled from 10× to 63×. The xenon flashlamp was mounted 2 inches from the specimen at 45°. One of the dual output ports of the microscope was connected to a CCD camera attached to the lifetime-gated imaging module. The other port was connected to a Nikon DS-5M-L1 digital camera (Melville, NY) for capturing white light images.

Lifetime gated imaging module—The imageX-nanoCCD lifetime-gated system, developed by the Photonic Research Systems Ltd. (Salford, Greater Manchester, UK), can distinguish fluorescent labels on the basis of their lifetime, down to 0.5 ns timing resolution. The system consists of a CCD camera, image-readout system and software package. The CCD camera has a resolution of 752×582 pixels at 14 bits with a chip size of 2/3 inch. The camera has 50% sensitivity between 430-730 nm, with the peak sensitivity at 550 nm, well suited to capture the green emission bands of terbium dipicolinate (λ_{em} = 544 nm). It is thermoelectronic and is cooled to 40°C below ambient temperature during operation to minimize noise due to dark current. The module also offers a TTL-compatible external trigger input to allow the triggering of camera gating circuitry by free-running laser pulses.

The software communicated with the CCD camera and xenon flashlamp by an external integrated timing circuitry housed in a control box. The control box was connected to the host computer *via* a parallel interface and was controlled from within the software to initiate a flash trigger sequence. Pulse width, light source frequency, integration and delay time are all parameters controllable in the software. The full frame readout is 0.8 second for medium resolution and 5 seconds for high resolution. The camera remains insensitive during the illumination period. After a set delay the camera becomes sensitive to light and images the signal emitted after this point until the end of the predetermined exposure time. These measures enable precise gating and significantly minimize problems caused by photobleaching.

Other optical components—Glass was eliminated from both the excitation side and sample because mid-UV is effectively blocked by glass. Quartz slides (75×25mm, ChemGlass, Inc., Vineland, NJ), quartz cover slips (19×19mm, ChemGlass, Inc., Vineland, NJ) and quartz elliptical lenses (LA4052, ThorLabs, Inc., Newton, NJ) were used on the excitation side. The two quartz elliptical lenses were mounted in front of the xenon flashlamp source to direct focused light onto the specimen more effectively. These antireflective film-coated lenses minimize the transmission attenuation factor down to 1%. The wide viewing angle of 65° in the horizontal plane and 40° in the vertical plane ensured even illumination on the specimen. Sample was prepared on a quartz microscope slide with a quartz cover slip to let UV light pass through. A highpass filter (03FCG067, Melles Griot, Carlsbad, CA) centered at 500 nm was placed along the light path on the emission side before reaching the microscope objective. It allows the green emission bands from terbium dipicolinate to pass through and rejects the UV excitation. Figure 3 shows the emission and excitation spectra of terbium dipicolinate, xenon flashlamp emission spectrum as well as the transmittance spectrum of the UV-blocking highpass filter, highlighting the spectral overlapping and a large shift in the absorption and emission peak of terbium dipicolinate.

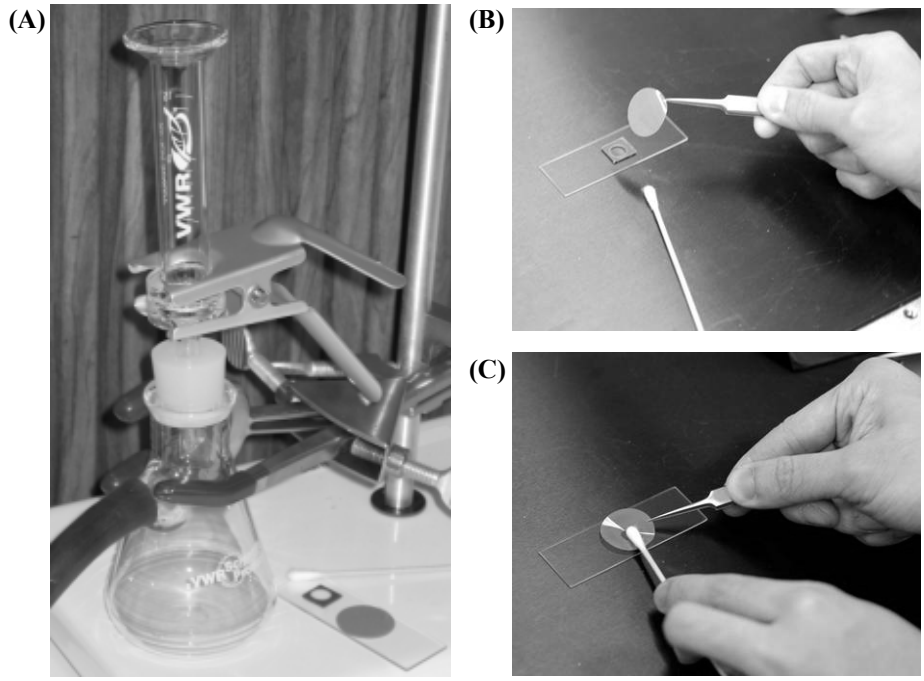

Figure 1: (A) Picture of the vacuum filtration unit. The tubing leading to the left is connected to a vacuum aspirator. A 0.2-µm polycarbonate filter (placed on the quartz microscope slide) is sandwiched between the filtration funnel and filter base for trapping spores. The silicone isolator (next to the filter) holds the solidified Tb^{3+} and L-alanine-doped agarose, mimicking a micro petri dish, as a germination substratum for spores. Vacuum filtration concentrates and immobilizes spores onto the membrane filter. (B, C) Spores are transferred onto agarose by streaking the membrane filter across the agarose surface, which will be ready for lifetime-gated imaging of germinating spores.

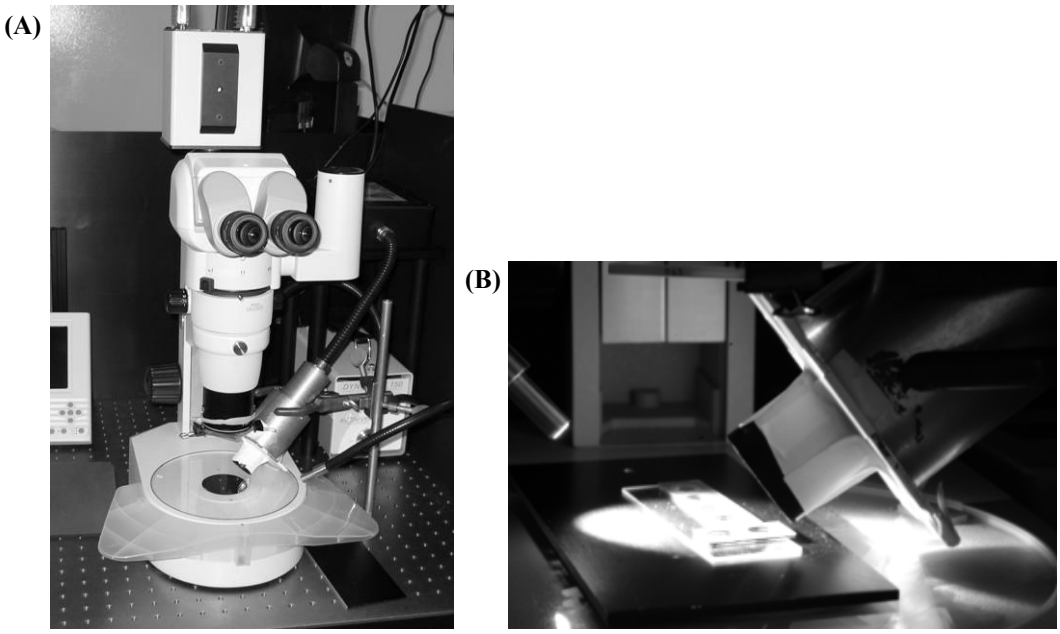

Figure 2: (A) Schematic of the setup of lifetime gated microscopy. The stereoscopic microscope provides sufficient room to accommodate the xenon flashlamp between the stage and objective, as well as a large field of view to facilitate counting. The xenon flashlamp is mounted at 45º to provide UV excitation at 300 Hz. A lifetime-gated CCD camera is attached to the microscope. The entire configuration is contained in an enclosure to exclude ambient light. (B) A close-up picture of the microscope. A number of silicone isolators containing Tb^{3+} and L-alanine-doped agarose are placed on a quartz microscope slide. The xenon flashlamp is mounted to the right of the picture, attached with two quartz elliptical lenses. The probe on the left provides bright field illumination.

Methods

Coupon precleaning and sterilization—2.25"×2.25" coupons were rinsed with both 18.2 MΩ-cm deionized water and acetone to remove surface impurities and stains. They were then cleaned with clean-room grade polyester wipes (BD Consumer Healthcare, Franklin Lakes, NY) saturated with 70% isopropyl alcohol. Each coupon was individually wrapped in a sterilization pouch (Fishbrand, Pittsburgh, PA) and autoclaved at 121°C for 15 minutes. Each autoclaved coupon was transferred aseptically to a disposable sterile plastic petri dish.

Coupon inoculation with spore suspension—The sterile coupons were inoculated with spores of *B. atrophaeus* ATCC 9372. 1 ml of spore suspension in methanol was pipetted aseptically evenly onto the coupons. Coupons with a concentration of 1.3×10^5 TSU/coupon (4000 TSU/cm^2) were inoculated with spores with lot number 1092371. Coupons with concentration of 5.0×10^4 TSU/coupon (1500 TSU/cm^2) and 7.0×10^3 TSU/coupon (200 TSU/cm^2) were inoculated with spores with lot number 1092411. Care was taken to spread the suspension evenly over the surface but not to drip over the edge. Coupons were air dried at room temperature inside petri dishes without controlling the humidity overnight.

Swab-rinse method—The procedures for sampling the coupons and processing the spores were similar to those described in NASA handbook 5340.1D (NHB 5340.1D) [35]. Sterile cotton applicators were used to sample the coupons (1 applicator per coupon). A single applicator was moistened in sterile water (Nanopure, 18 MΩ-cm) and the coupon was swabbed 3 times with the same cotton applicator. The coupon was rotated 90° after the first swab sampling, and 45° after the second swab sampling. The swab head was placed into 20 ml of sterile water and mixed by vortexing for 5 to 10 seconds. A total of 5 coupons were swabbed for each inoculum size. The applicator head in water was sonicated for 2 min at 25 kHz to recover spores. A 10 ml aliquot was removed and subjected to a heat shock (15 min at 80 ± 2°C). The remaining 10 ml was not subjected to the heat shock. A 5 ml aliquot of the heat

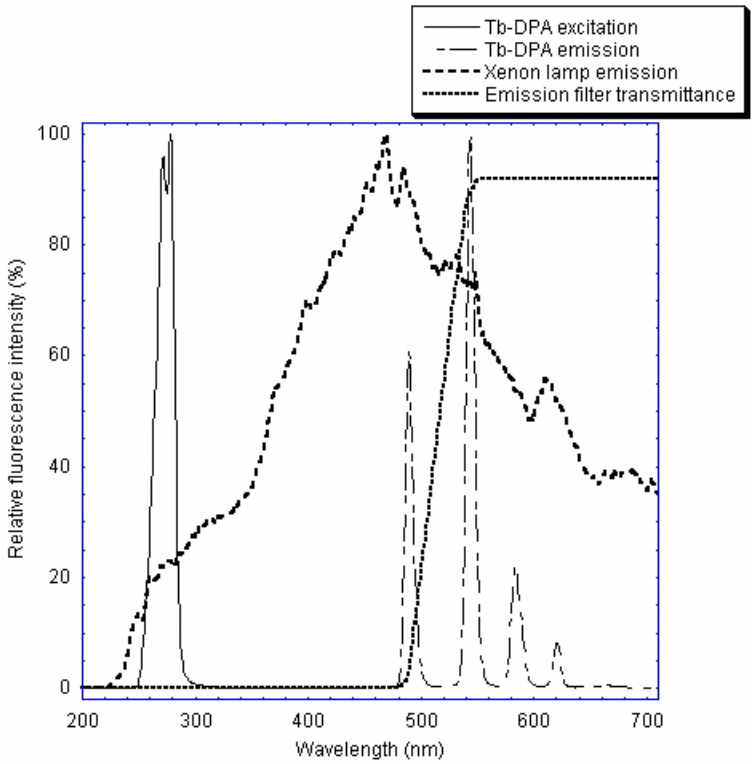

Figure 3: Spectral overlap of different spectra. Excitation spectrum of terbium dipicolinate (10 μM $TbCl_3$ + 62.5 nM DPA in 1 M sodium acetate buffer at pH 5.8) at 544 nm emission wavelength is shown in light solid line. Excitation spectrum of the same terbium dipicolinate solution at 278 nm excitation wavelength is shown in light broken line. The xenon flashlamp emission spectrum (heavy dashed line) is shown overlapping with Tb-DPA excitation band in the mid-UV 250 - 300 nm range. The xenon flashlamp provides a broadband emission wavelength from UV to IR, with peak intensity at 460 nm. Transmittance spectrum of the emission UV-blocking highpass filter (heavy densely-packed dashed line) shows that it can effectively prevent most of the UV excitation from entering the camera. In addition to this, a delay of 100-μs discriminates the long-lived Tb-DPA signal from the short-lived xenon excitation. Green luminescence exhibited by Tb-DPA can thus be imaged by the lifetime-gated CCD camera.

shock sample and a 5 ml aliquot of the non-heat shock sample were removed for use in RapidSSEA. The remaining 5 ml of the heat shock sample and the remaining 5 ml of the non-heat shock sample were used in the pour plate method of the NASA standard assay. Positive and negative controls were run for each set of coupons as necessary.

After the coupons were swabbed (or not swabbed as in the positive control), they were aseptically transferred to sterile 600 ml beakers. 40 ml of sterile water was added to the coupons. The coupons were sonicated for 2 min at 25 kHz to recover any spores that were remaining on the surface. A 10 ml aliquot was removed and subjected to a heat shock (15 min at 80 ± 2°C). A separate 10 ml aliquot was not subjected to the heat shock. A 5 ml aliquot of the heat shock sample and a 5 ml aliquot of the non-heat shock sample were removed for use in the lifetime gated imaging assay. The remaining 5 ml of the heat shock sample and the remaining 5 ml of the non-heat shock sample were used in the pour plate method of the NASA standard assay.

The samples were: 1) heat shock swab; 2) no heat shock swab; 3) heat shock coupon; 4) no heat shock coupon. The positive control consisted of: 1) heat shock coupon; 2) no heat shock coupon. Negative controls included: 1) TSA media controls (media only in the petri dish); 2) swab water control (water that the swab was dipped into before it was used to swab the coupon); 3) dilution water control (water that was used to make the dilutions before the recovered spores were diluted and plated). Figure 4 outlines the experimental procedure in a flowchart.

NASA standard assay—The following procedure was used to perform the pour plate method of the NASA standard assay (NHB 5340.1D). In the NASA standard assay of flight hardware for Planetary Protection implementation, it is usually not necessary to make dilutions of the samples before plating. However, some inoculum sizes in this experiment required that serial dilutions be made in order to The samples were stored at 4°C for about an hour after heat shock and before dilutions were made. Serial 10-fold dilutions were made for each sample. For the dilutions, 4.5 ml of water was used as the diluent. A 0.5 ml sample was removed and added to the diluent to produce a 10-fold serial dilution. The dilution range was 10^0 (undiluted) to a 10^{-5} dilution.

The pour plate method was used. 2 ml aliquots of the diluted samples were placed into 2 petri dishes (4 ml total). Approximately 25 ml of sterile, molten TSA was added to each plate and gently swirled. A mediaclave (Integra Biosciences, Chur, Switzerland) was used to dispense the sterile media. The plates were allowed to harden and were incubated at 32°C for 3 days. The colonies on the plates were counted after incubation.

RapidSSEA—RapidSSEA involves two parts, membrane filtration and lifetime-gated microscopy. Agarose was prepared at a ratio of 15 g/l in deionized water. Terbium (III) chloride and L-alanine were added to agarose such that the final concentrations was 100 μM and 10 mM, respectively [22,36,37]. The terbium- and L-alanine-doped agarose was sterilized in an autoclave at 121°C for 15 minutes. The final pH of the agarose was 5.5 ± 0.1, in good agreement with the reported optimal pH to detect DPA triggered terbium luminescence [30,33]. 80 μl of agarose was pipetted to fill up sterile silicone isolator on a quartz slide, mimicking a micro petri dish. The medium was ready for imaging after ~2 minutes of cooling and solidification.

1 ml aliquots from each sample were pipetted into a sterile filtration funnel connected to the membrane filtration unit. Spores and other particles larger than 0.2 μm were retained on the polycarbonate membrane filter as air was aspirated. The funnel was rinsed with 1 ml sterile water and was aspirated again. The membrane filter was then aseptically turned upside down and placed on top of the agarose substrate by a pair of sterile tweezers. The backside of the membrane filter was rubbed by a sterile cotton applicator to transfer spores and other trapped particles to agarose. The filter was swabbed against the substrate 3 times with the same cotton applicator. The swabbing direction was rotated 90° after the first transfer and 45° after the second transfer.

Depositing spores on agarose substrate triggered off the onset of germination. DPA release follows stage I germination within a timescale of minutes [22-24]. It took approximately 10 minutes to fully manifest DPA-sensitized terbium luminescence using RapidSSEA. An initial examination was made to ensure an even distribution of spores on the medium. Lifetime-gated images were captured with a 100-μs delay integrating for 2 milliseconds. 6 to 13 images were taken over different areas of the medium. GSU were visualized as bright spots in the image. Each image covers an actual agarose area of 3.2 mm^2 at 40× magnification. Results of these samples were reported as GSU per image. To estimate the total GSU in a 1-ml aliquot, GSU per image was averaged over the number of images taken and multiplied by a scaling factor of 12 (silicone isolator area = 38.5 mm^2). GSU were reported as too numerous to count if they exceeded 300 in a single image.

Enumeration of GSU was automated with a counting algorithm written in ImageJ (Rasband, 1997-2005), a public domain image processing API in Java, funded by the National Institutes of Health (NIH) [38]. A threshold was applied to discriminate bright spots caused by spores from the dim background.

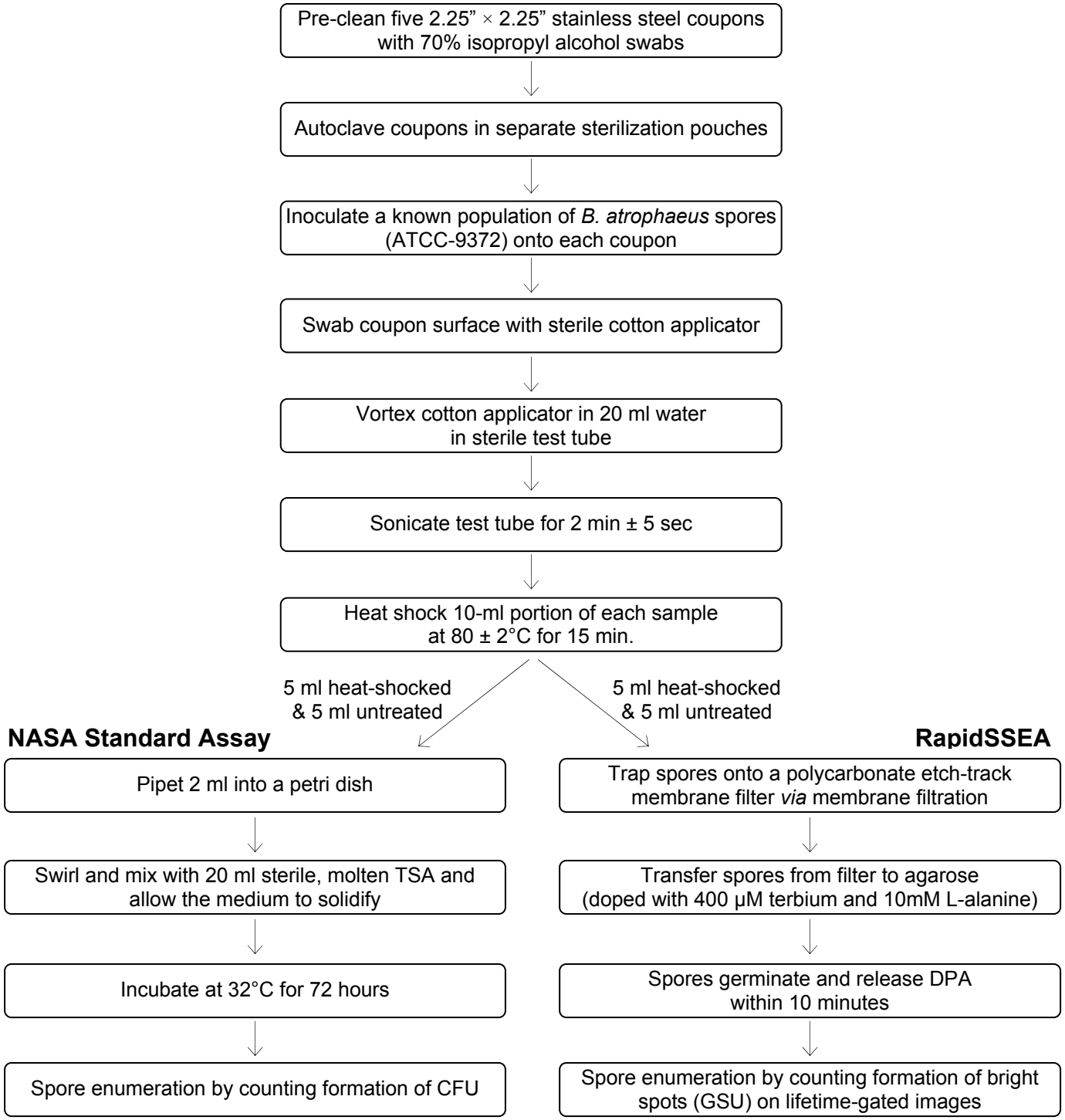

Figure 4: Flowchart of the experimental procedure. The pre-cleaning, inoculation and swab-rinse technique are common for both RapidSSEA and the NASA standard assay. Heat shock selects for bacterial spores over vegetative cells. Extractions are split into halves and subjected to both assays for parallel comparison. The traditional cultivation-based NASA standard assay uses TSA pour plating to assess cultivable spore population in terms of CFU in 3 days. RapidSSEA employs a time-resolved fluorescence-based technique to enumerate spores in less than 30 minutes in terms of GSU by inducing release of DPA *via* spore germination.

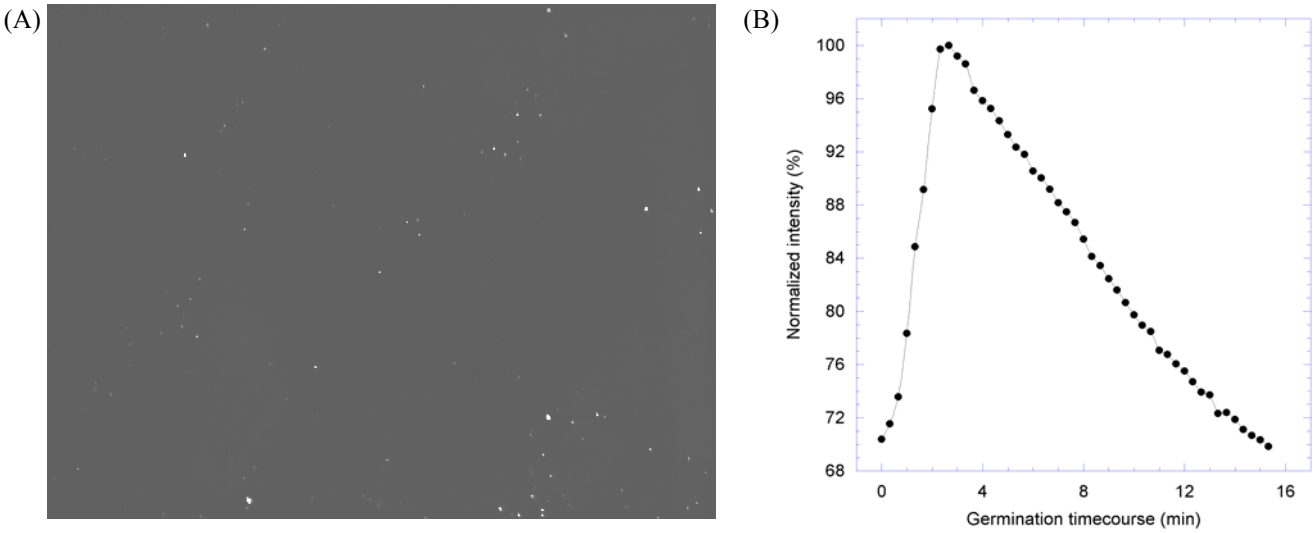

Figure 5: (A) Lifetime-gated image of germinated spores on terbium and L-alanine-doped agarose. The bright white spots in the image represent the presence of terbium dipicolinate halos surrounding each individual spore, signaling stage I of spore germination in which DPA is released. Each bright spot indicates a single germinating spore unit (GSU). (B) Intensity timecourse of a single germinating spore on terbium- and L-alanine-doped agarose. Spores were deposited on the agarose at time zero. The initial intensity rise was due to DPA release *via* germination into the local vicinity. Subsequent drop in intensity was due to continual diffusion of DPA across the gel-like agarose.

3. Results and Discussion

The process of germination releases DPA within a few minutes, which binds with Tb^{3+} to form a bright halo surrounding the spore that is approximately 10 times the diameter of the spore body. While 1-µm spores are not spatially resolved at the magnification provided by a stereoscopic microscope, we can easily image and count the bright spots that are generated when DPA combines with Tb^{3+} after DPA release during germination. Germinating spores manifest themselves as bright spots under a dark background when imaged with a lifetime-gated CCD camera at 63× under a stereoscopic microscope. An image of spores with 100-µs delay is shown in figure 5. Each of the bright spots constitutes a GSU.

Comparison of RapidSSEA and NASA Standard Assay

Stainless steel coupons inoculated with bacterial spores were sampled with cotton applicators and subsequently resuspended in water before being assayed by both RapidSSEA and the NASA standard assay. Five coupons each were inoculated with three different bacterial spore populations, which were determined to be (1) 1.3×10^5 TSU consisting of 53% GSU and 35% CFU, (2) 5.0×10^4 TSU consisting of 68% GSU and 24% CFU, and (3) 7.0×10^3 TSU consisting of 63% GSU and 24% CFU. TSU, GSU, and CFU populations were determined using hemocytometer counting under phase contrast microscope, RapidSSEA, and TSA spread plate counting, respectively.

Table 1 shows the comparison of spore recovery efficiency from stainless steel coupons using RapidSSEA and the NASA standard assay. Mean percentage recoveries are expressed in terms of GSU recovered with respect to GSU inoculated, CFU recovered with respect to CFU inoculated, and GSU and CFU recovered with respect to TSU inoculated. Spore populations were determined from both the cotton applicators that were used to swab the inoculated coupons and the swabbed coupons to determine spore populations remaining after swabbing.

RapidSSEA results show a GSU recovery of 62.2% ± 9.4% on cotton applicators; 30.4% ± 8.9% on swabbed coupons, while the NASA standard assay results show a CFU recovery of 27.0% ± 8.9% on cotton applicators; 25.6% ± 5.2% on swabbed coupons. Clearly, RapidSSEA is more effective in accounting for the spore population on stainless steel coupon surface than the NASA standard assay. GSU recovered from cotton applicators and swabbed coupons constitute nearly 100% of the initial inoculum size. In contrast, about 50% of the inoculated CFU remained unaccounted when the NASA standard assay was employed. The spore recovery percentage obtained by RapidSSEA in terms of CFU is consistent with the value reported by Rose [39]. Rose performed a similar experiment using cotton applicators for swabbing a spore-laden 2"×2" (5.1 cm × 5.1 cm) stainless steel coupon surface and cultivation-based

Table 1: Spore recovery efficiencies from stainless steel coupons using RapidSSEA and the NASA standard assay.

	Mean percentage recovery (standard deviation), N = 15[i]					
	%GSU recovery[ii]		%CFU recovery[iii]		%GSU, CFU recovery wrt TSU[iv]	
	Recovery from cotton applicator	Recovery from swabbed coupon	Recovery from cotton applicator	Recovery from swabbed coupon	Recovery from cotton applicator	Recovery from swabbed coupon
RapidSSEA	62.2 (9.4)	30.4 (8.9)	N/A	N/A	38.0 (5.8)	18.9 (5.3)
NASA standard assay	N/A	N/A	27.0 (8.9)	25.6 (5.2)	7.4 (2.7)	7.7 (1.3)

[I] Sampling population consists of 5 coupons inoculated with 1.3×10^5 TSU (53% GSU, 35% CFU), 5 coupons with 5.0×10^4 TSU (68% GSU, 24% CFU) and 5 coupons with 7.0×10^3 TSU (63% GSU, 24% CFU), as determined by hemocytometer counting under phase contrast microscopy, RapidSSEA and TSA spread plate counting, respectively.

[ii] Germinating spore unit (GSU) percentage recovery is computed by dividing GSU recovered *via* RapidSSEA by the total inoculated GSU. GSU recovery is not applicable for the NASA standard assay because it measures CFU.

[iii] Colony forming unit (CFU) percentage recovery is computed by dividing CFU recovered *via* the NASA standard assay by the total inoculated CFU. CFU recovery is not applicable for RapidSSEA because it measures GSU.

[iv] Total spore unit (TSU) percentage recovery is computed by dividing GSU recovered via RapidSSEA and CFU recovered *via* the NASA standard assay by the inoculated TSU, respectively.

assay to assess the population of *Bacillus anthracis*. Rose reported a spore recovery percentage of 27.7% ± 17.7%, in good agreement with our results, 27.0% ± 8.9%. Differences in the spore extraction process are noted between the two assays. Rose used 2 minutes of 10-sec burst vortex and 12 minutes of sonication at 42 kHz. The NASA standard assay used a 5-6 sec vortex and 2 min ± 5 sec sonication at 25 kHz.

Figure 6 shows a concentration breakdown of the GSU and CFU recoveries. The high coefficients of determination suggest a linear relationship between inoculum size and the number of spores recovered down to at least approximately 10^3 spores per coupon.

The last two columns in Table 1 express spore recovery percentages with respect to TSU. RapidSSEA reports a TSU recovery of 38.0% ± 5.8% on cotton applicators;

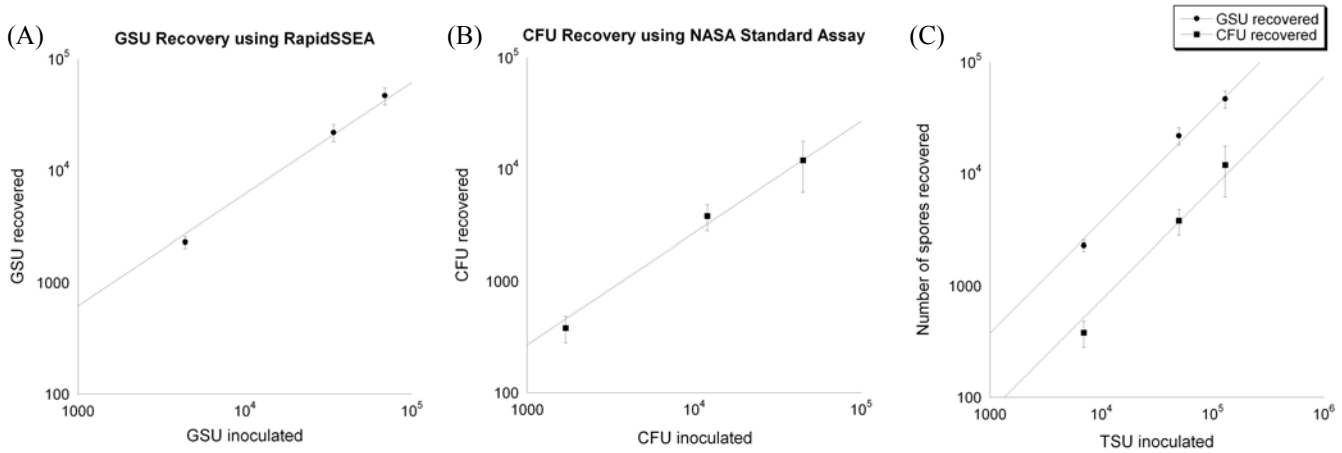

Figure 6: (A) GSU recovery using RapidSSEA. (B) CFU recovery using the NASA standard assay. Both data sets can be fitted with a linear plot with a close-to-unity coefficient of determination. (C) Comparison of TSU recovery percentages using both RapidSSEA and the NASA standard assay. Both data sets demonstrate a linear correlation between TSU and the number of GSU and CFU recovered, respectively.

18.9% ± 5.3% on swabbed coupons. The NASA standard assay reports a CFU recovery of 7.4% ± 2.7% on cotton applicators; 7.7% ± 1.3% on swabbed coupons. Figure 6C shows the TSU recovery percentage plots using both assays.

Difference in TSU recoveries sheds light on the viability of the actual bioburden on a surface because there is no universal growth media. Viable spores may not form colonies in one medium, but may do in another medium. Second, several spores may aggregate to give rise to a single colony, making this assay liable to underestimation. RapidSSEA does not suffer from these limitations as it detects for the release of DPA, a process during the early onset of spore germination. Therefore, non-cultivable sporeformers can be counted as long as they can germinate under the experimental conditions provided. The entire viable spore population, irrespective of cultivability, is the potential detection target. Although RapidSSEA does not reflects the actual bioburden accurately either, the complement of these two assays can definitely provide an upper and lower bound for viable spore population, which will be a useful piece of information for determining total biomass on spacecraft hardware surfaces.

The NASA standard assay entails much more manpower, resources and time than RapidSSEA. Owing to the long 72-hour incubation time, quick experiments cannot practically be taken to pre-determine the approximate spore population and species. Plates may have to be prepared twice or thrice as many to cover several dilution factors so that CFU on petri dishes fall within countable range (30 – 300 CFU). Different types of growth media may have to be prepared to maximize the chance of cultivability, which further doubles or triples the total number of plates, manpower and time. In this study, for instance, almost 300 plates were prepared to examine a known concentration of a single spore species on 5 coupons. Examining environmental samples with unknown spore concentration and species adds to its complexity. Disposal of bulky used plates poses serious environmental implications, too. RapidSSEA, on the other hand, is a fast, less expensive, less labor-intensive and more environmental-friendly method. Onset of spore germination and DPA release induced by L-alanine occur on a timescale of minutes. The overall RapidSSEA procedure takes less than 30 minutes to evaluate bioburden on a surface, hastened by automatic spore enumeration realized with a computer software algorithm. A single type of medium is sufficient for RapidSSEA because L-alanine is a universal germinant to spore-forming species. Each 9-mm silicone isolator can be filled up by 80 µl of molten agarose compared with the 10-cm, 20-ml petri dishes. This 250-time volume difference and size difference save a lot of resources and mitigates the problem of waste disposable.

A considerable amount of spores remain on the coupons after swabbing. Spores are known to attach to surfaces to a greater extent than vegetative cells [40]. Adhesion of *Bacillus* spores is enhanced because of the hydrophobic property of stainless steel coupon [41,42]. Spore attachment is a two-stage process [43]. The first stage is characterized by reversible weak electrostatic forces holding between spores and surfaces. The second stage is an irreversible step in which exopolysaccharides are produced for firm substrate attachment [44]. Biofilm formation follows this second stage under favorable conditions. It requires dedicated procedures to clean a spore-laden surface to sterility [45]. In this study, *B. atrophaeus* spores inoculated onto stainless steel coupons might have reached the second stage of adhesion during overnight drying. This strong spore adhesion might account for residual spores on swabbed coupons. In addition to this, a fraction of spores might be lysed by sonication [46]. Some stainless steel coupons were observed to have surface scratches, which might provide shelters for spores and hinder them from being recovered by cotton applicators [47].

No significant difference is observed between samples with and without heat shock treatment in coupons inoculated with 1.3×10^5 TSU. With heat shock, RapidSSEA reports a GSU percentage recovery of 71.3% ± 15.5% on cotton applicators and 18.2% ± 8.9% on swabbed coupons. Without heat shock, RapidSSEA reports a GSU percentage recovery of 68.7% ± 11.8% on cotton applicators and 24.9% ± 12.0% on swabbed coupons. The close proximity of heat shock and non heat shock results are also verified in the NASA standard assay. With heat shock, the NASA standard assay reports a CFU percentage recovery of 26.0% ± 19.3% on cotton applicators and 41.3% ± 2.5% on swabbed coupons. Without heat shock, the NASA standard assay reports a CFU percentage recovery of 26.8% ± 12.9% on cotton applicators and 42.7% ± 3.1% on swabbed coupons. In both assays, heat shock and non heat shock results are very close, showing the microorganisms being examined are primarily heat-shock survivors, i.e. bacterial spores.

Concentration Dependence of RapidSSEA

Figure 7 shows the results of a concentration dependence experiment using RapidSSEA. Stainless steel coupons were seeded with spores of inoculum sizes from 10^5 GSU down to 10 GSU. The graph leveled off at a background count of 326 GSU/coupon, i.e. ~11 GSU/cm^2. The limit of detection at 3 sigma was found to be around 5000 GSU/coupon, i.e. ~150 GSU/cm^2. False positives resembling bright spots might be due to undissolved terbium (III) chloride pellets present in the solid agarose matrix, fallout spores or long-lived airborne interferents. This background luminescence issue had to be characterized and resolved in future assay development. 5000 spores/coupon was too high a detection limit to examine bioburden on spacecraft hardware. Spacecraft surfaces typically contain 0 to 4 spore-formers per cm^2 during assembly in clean-rooms [47,48]. Depending on the planetary protection requirements for the specific mission, the total number of spores on the entire spacecraft may only be 1×10^5 spores or less. RapidSSEA needs to be improved on the low concentration regime to be able to detect <10 spores per sample.

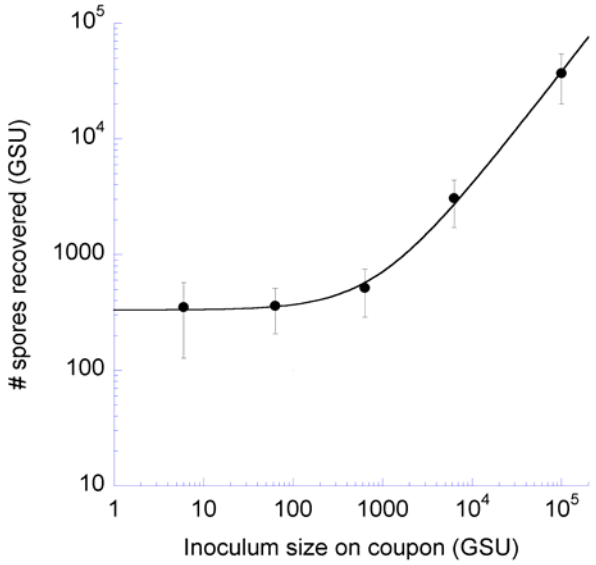

Figure 7: Concentration dependence experiment of spore recovery using RapidSSEA. Both axes are plotted in logarithmic scale. The curve levels off at low inoculum sizes and intercepts the y-axis at 326, which represents the baseline value.

Future Work

The reported represents the first iteration of validating the novel RapidSSEA approach for quantifying the bacterial spore bioburden on spacecraft surface. Many improvements are envisioned by the authors.

The current lifetime-gated microscopy configuration is amenable to higher resolution with laser-coupled compound microscope. A 266-nm Nd:YAG laser will be used to provide monochromatic, coherent and high power UV excitation for terbium dipicolinate photoluminescence detection. Bright field, darkfield, and phase contrast images can also be taken with the compound microscope. Germination of bacterial spores can be characterized and observed by a phase-bright to phase-dark transition under the microscope. Visualization of this transition confirms that germination of the spores has occurred. The aforementioned future work is envisioned to improve the detection limit to less than 10 spores/coupon.

The current spore counting algorithm is based on pixel intensity values. A gray-scale intensity value is defined to be a threshold, above which GSU lies and below which encompasses the background. Lifetime-gated image can be segmented and thresholded because of the bimodal distribution of the intensity histogram, owing to the brightly luminescent GSU and the relatively dark background. The size of terbium dipicolinate halo is assumed not to exceed 10 μm^2, which is set to be the upper bound for the unit counting size in the algorithm. GSU in a lifetime-gated image can be enumerated within seconds of full automation. In future image processing, adaptive thresholding may be used and spores will be identified by their characteristic intensity profile, featuring an initial sharp rise (release of DPA) followed by a gradual drop (diffusion of DPA across agarose), as illustrated in figure 5B.

4. CONCLUSIONS

A side-by-side comparison has been performed between RapidSSEA and the NASA standard assay to evaluate the efficiency of *Bacillus atrophaeus* spore recovery on 2.25" ×2.25" stainless steel coupons. Cotton applicators are used to swab coupons inoculated with 1.3×10^5 TSU, 5.0×10^4 TSU and 7.0×10^3 TSU. RapidSSEA has proved to be a rapid method, entailing <30 minutes, to assess the germinated spore population on coupons with a recovery efficiency of 62.2% ± 9.4% based on total GSU inoculum; 38.0% ± 5.8% based on TSU inoculum. The NASA standard assay completed the same task in 3 days with a recovery efficiency of 27.0% ± 8.9% based on total CFU inoculum; 7.5% ± 2.7% based on TSU inoculum. RapidSSEA is preferable to the NASA standard assay in terms of recovery efficiency, speed, labor force and resources. The two assays complement each other to establish the upper and lower bounds for viable spore populations. However, high background counts in RapidSSEA highlight the need to improve this lifetime gated microscopy assay so that the current 5000 spores/coupon detection limit could be pushed lower for more rigorous validation of sterilization.

5. ACKNOWLEDGMENTS

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REFERENCES

[1] B. D. Church and H. Halvorson , "Dependence of the heat resistance of bacterial endospores on their dipicolinic acid content," Nature 183, 4654 124-125, 1959.

[2] A. F. Byrne, T. H. Burton, et al., "Relation of dipicolinic acid content of anaerobic bacterial endospores to their heat resistance," Journal of Bacteriology 80(1), 139-140, 1960.

[3] P. E. Berg and N. Grecz, "Relationship of dipicolinic acid content in spores of Bacillus cereus to ultraviolet and gamma radiation resistance," Journal of Bacteriology 103(2), 517-519, 1970.

[4] T. A. Roberts and Hitchins, A. D, "Resistance of spores," p. 611–670, in Gould, G. W. and Hurst, A. (ed.), The Bacterial Spore. New York: Academic Press, 1969.

[5] P. Setlow, “Bacterial spore resistance,” p. 217–230, in Storz, G. and Hengge-Aronis, G. (ed.), Bacterial stress responses. Washington, D.C.: American Society for Microbiology, 1999.

[6] K. Fox and B. D. Eder. "Comparison of survivor curves of Bacillus subtilis spores subjected to wet and dry heat," Journal of Food Science 34, 518-521, 1969.

[7] H. W. Jannasch, C. O. Wirsen, et al., "Comparative physiological studies on hyperthermophilic archaea isolated from deep-sea hot vents with emphasis on Pyrococcus strain GB-D," Applied and Environmental Microbiology 58, 3472-3481, 1992.

[8] E. Y. Wuytack, S. Boven, S. et al., "Comparative study of pressure-induced germination of Bacillus subtilis spores at low and high pressures," Applied and Environmental Microbiology 64(9), 3220-3224, 1998.

[9] J. Koike, T. Oshima, et al., "Survivor rates of some terrestrial microorganisms under simulated space conditions," Advances in Space Research 12(4), 271-274, 1992.

[10] W. L. Nicholson, N. Munakata et al., "Resistance of Bacillus endospores to extreme terrestrial and extraterrestrial environments," Microbiology and Molecular Biology Reviews 64(3), 548-572, 2000.

[11] R. J. Cano and M. K. Borucki, "Revival and identification of bacterial spores in 25- to 40-million-year-old Dominican amber," Science 268(5213), 1060-1064, 1995.

[12] R. H. Vreeland, W. D. Rosenzweig et al., "Isolation of a 250 million-year-old halotolerant bacterium from a primary salt crystal," Nature 407(6806), 897-900, 2000.

[13] R. Dart, Microbiology for the Analytical Chemist, Cambridge, UK: The Royal Society of Chemistry, 1996.

[14] W. Keeton, Biological Science, New York: W. W. Norton & Co., 1980.

[15] G. W. Gould and A. Hurst, The Bacterial Spore, New York: Academic Press, 1969.

[16] W. M. Napier, "A mechanism for interstellar panspermia," Monthly Notices of the Royal Astronomical Society 384(1), 46-51, 2004.

[17] Office of Space Science, National Aeronautics and Space Administration, Planetary Protection Provisions for Robotic Extraterrestrial Missions, Washington, D.C, 1999.

[18] S. S. Board and N. R. Council, Preventing the Forward Contamination of Europa, Washington, D.C.: National Academy Press, 2000.

[19] R. Amann, I., W. Ludwig, et al., "Phylogenetic identification and in situ detection of individual microbial cells without cultivation," Micriobiological Reviews 59(1), 143-169, 1995.

[20] V. Torsvik, J. Goksoyr, et al., "High Diversity in DNA of Soil Bacteria," Applied and Environmental Microbiology 56(3), 782-787, 1990.

[21] J. G. Jones, "Effect of Environmental-Factors on Estimated Viable and Total Populations of Planktonic Bacteria in Lakes and Experimental Enclosures," Freshwater Biology 7(1), 67-91, 1977.

[22] T. Hashimoto, W. R. Frieben, et al., "Microgermination of Bacillus cereus spores," Journal of Bacteriology 100(3), 1385-1392, 1969.

[23] P. Setlow, "Spore germination," Current Opinion in Microbiology 6(6), 550-556, 2003.

[24] S. J. Foster and K. Johnstone, "Pulling the trigger, the mechanism of bacterial spore germination," Molecular Microbiology 4(1), 137-141, 1989.

[25] A. Moir and D. A. Smith, "The genetics of bacterial spore germination," Annual Review of Microbiology 44, 531-553, 1990.

[26] L. E. Sacks, "Chemical Germination of Native and Cation-Exchanged Bacterial-Spores with Trifluoperazine," Applied and Environmental Microbiology 56(4), 1185-1187, 1990.

[27] A. Vaid and A. H. Bishop. "The destruction by microwave radiation of bacterial endospores and amplification of the released DNA," Journal of Applied Microbiology 85(1), 115-122, 1998.

[28] E. D. Lester and A. Ponce, "An anthrax "smoke" detector, Online monitoring of aerosolized bacterial spores," IEEE Engineering in Medicine and Biology Magazine 21(5), 38-42, 2002.

[29] D. L. Rosen, "Bacterial endospore detection using photoluminescence from terbium dipicolinate," Reviews in Analytical Chemistry 18(1-2), 1-21, 1999.

[30] A. A. Hindle and E. A. H. Hall, "Dipicolinic acid (DPA) assay revisited and appraised for spore detection," Analyst 124(11), 1599-1604, 1999.

[31] E. D. Lester, B. Gregory, et al., "A second-generation anthrax ‘smoke detector’," IEEE Engineering in Medicine and Biology Magazine 23(1), 130-135, 2004.

[32] D. L. Rosen and S. Niles, "Bacterial endospore detection using photoluminescence from terbium dipicolinate," Reviews in Analytical Chemistry 18(1-2), 1-21, 1999.

[33] G. Jones, V. I. Vullev, "Medium effects on the photophysical properties of terbium(III) complexes with pyridine-2,6-dicarboxylate," Photochemical & Photobiological Sciences 1(12), 925-933, 2002.

[34] D. Fritze, R. Pukall, "Reclassification of bioindicator strains Bacillus subtilis DSM 675 and Bacillus subtilis DSM 2277 as Bacillus atrophaeus," International Journal of Systematic and Evolutionary Microbiology 51, 35-37, 2001.

[35] Anonymous, "NASA standard procedures for the microbiological examination of space hardware, NHB 5340.1D," Jet Propulsion Laboratory Communication, 1980.

[36] C. R. Woese, H. J. Morowitz, et al., "Analysis of action of L-alanine analogues in spore germination," Journal of Bacteriology 76(6), 578-588, 1958.

[37] G. S. Stewart, K. Johnstone, et al., "Commitment of bacterial spores to germinate. A measure of the trigger reaction," Biochemical Journal 198(1), 101-106, 1981.

[38] W. S. Rasband, ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, U. S. A., http,//rsb.info.nih.gov/ij/, 1997-2005.

[39] L. Rose, B. Jensen, et al., "Swab materials and Bacillus anthracis spore recovery from nonporous surfaces," Emerging Infectious Diseases 10(6), 1023-1029, 2004.

[40] U. Husmark and U. Ronner, "The influence of hydrophobic electrostatic and morphological properties on the adhesion of Bacillus spores," Biofouling 5, 335-344, 1992.

[41] U. Ronner, U. Husmark, et al., "Adhesion of bacillus spores in relation to hydrophobicity," Journal of Applied Bacteriology 69(4), 550-556, 1990.

[42] K. Weincek, N. A. Klapes, et al., "Adhesion of Bacillus spores to inanimate materials, effects of substratum and spore hydrophobicity," Biofouling 3, 139-149, 1991.

[43] K. C. Marshall, R. Stout, et al., "Mechanism of the initial events in the sorption of marine bacteria to surfaces," Journal of General Microbiology 68, 337-348, 1971.

[44] E. A. Zottola, "Characterization of the attachment matrix of Pseudomonas fragi attached to non-porous surfaces," Biofouling 5, 37-55, 1991.

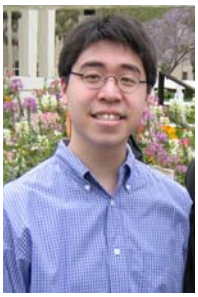
[45] M. T. La Duc, W. Nicholson, et al., "Microbial characterization of the Mars Odyssey spacecraft and its encapsulation facility," Environmental Microbiology 5(10), 977-985, 2003.

[46] M. T. Taylor, P. Belgrader, et al., "Lysing bacterial spores by sonication through a flexible interface in a microfluidic system," Analytical Chemistry 73(3), 492-496, 2001.

[47] K. Venkateswaran, S. Chung et al., "Evaluation of various cleaning methods to remove bacillus spores from spacecraft hardware materials," Astrobiology 4(3), 377-90, 2004.

[48] K. Venkateswaran, M. Satomi, et al., "Molecular microbial diversity of a spacecraft assembly facility," Systematic and Applied Microbiology 24(2), 311-320, 2001.

BIOGRAPHY



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