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TITLE:

Visualization of Germinosomes and the Inner Membrane in *Bacillus subtilis* Spores

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SHORT ABSTRACT :

Germinant receptor proteins cluster in 'germinosomes' in the inner membrane of *Bacillus subtilis* spores. We describe a protocol using super resolution microscopy and fluorescent reporter proteins to visualize germinosomes. The protocol also identifies spore inner membrane domains that are preferentially stained with the membrane dye FM4-64.

LONG ABSTRACT:

The small size of spores and the relatively low abundance of germination proteins, cause difficulties in their microscopic analyses using epifluorescence microscopy. Super-resolution three-dimensional Structured Illumination Microscopy (3D-SIM) is a promising tool to overcome this hurdle and reveal the molecular details of the process of germination of *Bacillus subtilis* (*B. subtilis*) spores. Here, we describe the use of a modified SIMcheck (ImageJ)-assistant 3D imaging process and fluorescent reporter proteins for SIM microscopy of *B. subtilis* spores' germinosomes, cluster(s) of germination proteins. We also present a (standard)3D-SIM imaging procedure for FM4-64 staining of *B. subtilis* spore membranes. By using these procedures, we obtained unsurpassed resolution for germinosome localization and show that > 80% of *B. subtilis* KGB80 dormant spores obtained after sporulation on defined minimal MOPS medium have one or two GerD-GFP and GerKB-mCherry foci. Bright foci were also observed in FM4-64 stained spores' 3D-SIM images suggesting that inner membrane lipid domains of different fluidity likely exist. Further studies that use double labeling procedures with membrane dyes and germinosome reporter proteins to assess co-localization and thus get an optimal overview of the organization of *Bacillus* germination proteins in the inner spore membrane are possible.

INTRODUCTION:

Spores of the orders Bacillales and Clostridiales are metabolically dormant and extraordinarily resistant to harsh decontamination regimes, but unless they germinate, cannot cause deleterious effects in humans[1]. In nutrient germinant triggered germination of *Bacillus subtilis* (*B. subtilis*) spores, the initiation event is germinant binding to germinant receptors (GRs) located in the spore's inner membrane (IM). Subsequently, the GRs transduce signals to the SpoVA channel protein also located in the IM. This results in the onset of the exchange of spore core pyridine-2,6-dicarboxylic acid (dipicolinic acid; DPA; comprising 20% of spore core dry wt) for water via the SpoVA channel. Subsequently, the DPA release triggers the activation of cortex peptidoglycan hydrolysis, and additional water uptake follows[2-4]. These events lead to mechanical stress on the coat layers, its subsequent rupture, the onset of outgrowth and, finally, vegetative growth. However, the exact molecular details of the germination process are still far from resolved.

A major question about spore germination concerns the biophysical properties of the lipids surrounding the IM germination proteins as well as the IM SpoVA channel proteins. This largely immobile IM lipid bilayer is the main permeability barrier for many small molecules, including toxic chemical preservatives, some of which exert their action in the spore core or vegetative cell cytoplasm[5,6]. The IM lipid bilayer is likely in a gel state, although there is a significant fraction of mobile lipids in the IM[5]. The spore's IM also has the potential for significant expansion[5]. Thus, the surface area of the IM increases 1.6-fold upon germination without additional membrane synthesis and is accompanied by the loss of this membrane's characteristic low permeability and lipid immobility[5,6].

While the molecular details of the activation of germination proteins and organization of IM lipids in spores are attractive topics for study, the small size of *B. subtilis* spores and the relatively low abundance of germination proteins, pose a challenge to microscopic analyses. Griffiths et al. compelling epifluorescence microscope evidence, using fluorescent reporters fused to germination proteins, suggests that in *B. subtilis* spores the scaffold protein GerD organizes three GR subunits (A, B and C) for the GerA, B and K GRs, in a cluster[7]. They coined the term 'germinosome' for this cluster of germination proteins and described the structures as ~300 nm large IM protein foci[8]. Upon initiation of spore germination, fluorescent germinosome foci ultimately change into larger disperse fluorescent patterns, with >75% of spore populations displaying this pattern in spores germinated for 1 h with L-valine[8]. Note that the paper mentioned above used averaged images from dozens of consecutive fluorescent pictures, to gain statistical power and overcome the hurdle of low fluorescent signals observed during imaging. This visualization of these structures in bacterial spores was at the edge of what is technically feasible with classical microscopic tools and neither an evaluation of the amount of foci in a single spore nor their more detailed subcellular localization was possible with this approach.

Here, we demonstrate the use of Structured Illumination Microscopy (SIM) to obtain a detailed visualization and quantification of the germinosome(s) in spores of *B. subtilis*, as well as of their IM lipid domains[9]. The protocol also contains instructions for the sporulation, slide preparation and image analysis by SIMcheck (v1.0, an imageJ plugin) as well as ImageJ[10-12].

PROTOCOL:

1. *B. subtillis* sporulation (timing: 7 days before microscopic observation)

1.1. Day 1

1.1.1. Streak a bacterial culture on a Luria-Bertani Broth (LB) agar plate (1% tryptone, 0.5% yeast extract, 1% NaCl, 1% agar)[13] and incubate overnight at 37 °C to obtain single colonies. Use the *B. subtilis* KGB80 (PS4150 *gerKA gerKC gerKB-mCherry cat, gerD-gfp kan*) strain and its parent background strain *B. subtilis* PS4150 (PS832 *ΔgerE::spc, ΔcotE::tet*) as described previously[7].

NOTE: The use of spores with the *cotE gerE* background is essential in germinosome visualization, in order to minimize the autofluorescence of the spore coat layers[7].

1.1.2. Sterilize all media, tubes, pipets and other culture materials to be used with appropriate methods.

1.2. Day 2

1.2.1. Inoculate a single colony into 5 mL of LB medium early in the morning and incubate the culture under continuous agitation in a screw cap tube at 200 rpm/min and 37 °C until the OD_{600} reaches 0.3-0.4 (approximately 7 h).

1.2.2. Make 500 mL of MOPS medium (pH 7.5) containing 3 nM $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$, 0.4 µM H_3BO_3, 30 nM $CoCl_2\cdot 6H_2O$, 10 nM $CuSO_4\cdot 5H_2O$, 10 nM $ZnSO_4\cdot 7H_2O$, 1.32 mM K_2HPO_4, 0.4 mM $MgCl_2\cdot 6H_2O$, 0.276 mM K_2SO_4, 0.01 mM $FeSO_4\cdot 7H_2O$, 0.14 mM $CaCl_2\cdot 2H_2O$, 80 mM MOPS, 4 mM Tricine, 0.1 mM $MnCl_2\cdot 2H_2O$, 10 mM D-glucose-monohydrate, and 10 mM NH_4Cl.

1.2.3. Pof repare in screw cap tubes 10^{-1}- 10^{-7} serial dilutions of the LB culture in 5 mL MOPS medium each and incubate the cultures overnight under continuous agitation at 200 rpm/min and 37 °C.

NOTE: The serial dilutions prepared here aim to obtain a dilution in early exponential phase in the next morning. This step and the next step are necessary to allow the cells to adapt to the MOPS buffered growth medium.

1.3. Day 3

1.3.1 Select one of the MOPS dilutions with an OD_{600} of 0.3-0.4. Inoculate 0.2 mL of the culture to pre-warmed (37 °C) 20 mL of MOPS medium in a conical 250 mL flask and incubate under continuous agitation until the OD_{600} reaches 0.3-0.4 (~7 h).

1.3.2 Inoculate the MOPS based sporulation medium (250 mL) with 1% (v/v) pre-culture from step 1.3.1 and incubate for 3 days at 37 °C in a conical liter flask under continuous agitation. For FM4-64 staining of PS4150 spores, add 2 µg/mL FM4-64 probe (see **Table of**

Materials) to the sporulation medium 1 or 2 h after reaching the peak OD_{600} value of vegetative growth (generally approximately 2) and allow the culture to subsequently sporulate whilst protecting it from light[5].

1.4. Day 7: Harvesting of spores

1.4.1. Determine the sporulation yield (spores vs vegetative cells) using phase contrast microscopy at 100X magnification to distinguish the phase bright spores from phase dark cells and possibly non-mature spores. A 90 % phase bright spore yield is expected.

1.4.2. Pellet the spores at 4270 x *g* for 15 min at 4 °C in round bottom centrifuge tubes. Wash the spore pellet 2-3 times with 40 mL of sterile ultra-pure Type 1 demineralized water in 50 mL conical centrifuge tubes (see **Table of Materials**). Spin-down at each wash at 4270 x *g* for 15 min (4 °C).

1.5. Day 7: Spore purification

1.5.1 Spore purification: Suspend the spore pellet in 750 µL of 20% nonionic density gradient medium (see **Table of materials**) and load onto 800 µL of 50% nonionic density gradient medium in sterilized microcentrifuge tubes. Centrifuge for 60 min at 21500 × *g*. The pellet obtained contains the free spores. Suspend the spore pellet in 200 µL of 20% nonionic density gradient medium and load onto 1 mL of 50% nonionic density gradient medium in a microcentrifuge tube, and centrifuge for 15 min at 21500 × *g*.

1.5.2 Washing the final spore preparation and storage: The pellet obtained contains the purified dormant spores. Wash the spore pellet 2-3 times with 1.5 mL of sterile ultra-pure Type 1 water (see **Table of Materials**) and spin-down in between at 9560 × *g* for 15 min at 4 °C. Finally, suspend the pellet in sterile ultra-pure Type 1 water to a final OD_{600} of approximately 30. Aliquots of the spores can be stored at -80 °C for 8 weeks[14].

2. Decoating

2.1. Treat PS4150 spores with 0.1 M NaCl/0.1 M NaOH/1% sodium dodecyl sulfate (SDS)/0.1 M dithiothreitol (DTT) at 70 °C for 1 h. Wash the spores 10 times with sterilized ultra-pure Type 1 water[15,16]. By doing so, any adsorbed FM4-64 probe in spore's outer membrane and outer layers will be removed.

3. Coverslip and slide Preparation[11] (timing: 1 h before observation)

3.1. Pre-clean high precision coverslips (see **Table of materials**) with 1 M HCl for 30 min in a gently shaking water bath. Wash the coverslips twice in ultra-pure Type 1 water, and store them in 100% (vol/vol) EtOH. Let them dry and verify their clarity before use. Pre-clean the glass slides in 70% EtOH. Let them dry and verify their clarity before use.

4. Sampling fluorescent microspheres or spores in the gene frame slide[10] (timing 15 min)

4.1. Pre-warm two 70% EtOH cleaned and air dried glass slides (see **Table of materials**) for several seconds on a 70 °C heating block, drop 65 µL of sterilized 2% agarose held at 70 °C on top of one glass slide and place the other glass slide on top to spread the agarose between the slides. The agarose patch will dry in approximately 5 min.

4.2. Cut the agarose patch into a 1 x 1cm section after removing one of the glass slides, add 0.4 µL of sample (fluorescent microspheres or spores of ~10^8/mL), and transfer the patch onto the high precision coverslip by placing the coverslip onto the patch and sliding it off.

4.3. Fix a Gene Frame (1.5 × 1.6 cm, 65 µL) onto the dried slide, onto which the coverslip is placed closing all corners of the frame, thus completing the slide for use in microscopy.

5. Imaging[11, 17](timing 1 h)

5.1. Capture the transmission and fluorescence images of spores as well as a mixture of red and yellow-green carboxylate-modified fluorescent microspheres on a Structured Illumination Microscope (see **Table of materials**) equipped with a 100X oil objective (Numerical Aperture=1.49), a CCD camera and image analysis software (see **Table of materials**). Generate all images at room temperature without the disturbance of ambient light. Make sure to always clean the 100X objective and the slide with 75% ethanol before imaging.

5.2. Focus on 100 nm (diameter) fluorescent microspheres and optimize the point spread function (psf) by adjusting the correction ring on the 100X objective until a symmetric psf is obtained, thus minimizing blurring of the image. The psf is the impulse response or the response of an imaging system to a point source or point object.

5.3. Select a field of view with approximately 10 round fluorescent microspheres. Apply a grating focus adjustment for both 561 nm and 488 nm excitation wave lengths as the guide for the image analysis software.

5.4. Focus the spores with the transmission light and capture a transmission light image in the 16× average mode with 200 ms exposures for each image.

5.5. Capture 3D-SIM raw fluorescent images of the spores with the illumination mode “3D-SIM”, the camera settings to readout mode Electron Multiplying (EM) Gain 10MHz at 14-bit, and EM gain at 175. Excite the FM4-64 probe in PS4150 spores with 561 nm laser light at 20% laser power, and an illumination time of 400 ms.

5.6 Excite the GerKB-mCherry and GerD-GFP in KGB80 spores with, respectively, 561 nm laser light at 30% laser power for 1 s, and 488 nm laser light at 60% laser power for 3 s. Z-Stack settings are in the top to bottom mode, 0.2 µm/ step, 7 steps and 20 steps for germinosome and IM analysis, respectively.

NOTE: These laser parameters were applied in order to assure a maximum brightness value of the histogram window of around 4000.

6. Reconstruct 3D-SIM raw images of FM4-64 stained PS4150 spores

6.1. Perform the N-SIM Slice Reconstruction for the FM4-64 stained PS4150 spores' raw data. Click the **Param** button for **Reconstruct Slice** on the N-SIM pad tab sheet to open the N-SIM Slice Reconstruction window.

6.2. Set the reconstruction parameters in the **N-SIM Slice Reconstruction** window. To get perfect reconstructed images, follow the suggestion of the N-SIM instructions and click on the appropriate controls in the **N-SIM Slice Reconstruction** window to set the **Illumination Modulation Contrast (IMC)** to **Auto**, **High Resolution Noise Suppression (HRNS)** to 1.00 and **Out of the Focus Blur Suppression (OFBS)** to 0.05 as starting points.

6.3. Click the **Reconstruct Slice** button in the **N-SIM Slice Reconstruction** window to reconstruct the image. Evaluate the quality of the reconstected images by the Fast Fourier Transformed (FFT) images and reconstruction score, which display after reconstruction[12].

6.4. Adjust the **HRNS** from 0.10 to 5.00 and **OFBS** from 0.01 to 0.50 by clicking on the appropriate controls in the **N-SIM Slice Reconstruction** window until the best parameter settings are obtained.

6.5. Click the **Apply** button in the **N-SIM Slice Reconstuction** window to apply changed parameters. Click **Close** button to close the window.

6.6. Make a FM4-64 stained PS4150 spores raw image active and click the **Reconstruct Slice** button on the **N-SIM Pad** tab sheet to execute **Slice Reconstruction**. Save the reconstructed image.

7. Image analysis.

7.1. Convert Pseudo-Widefield images of the KGB80 germinosome

7.1.1. Convert 3D-SIM raw images of KGB80 into Pseudo-Widefield images by activating with a left click the ImageJ plugin **SIMcheck** utility **Raw Data SI** to **Pseudo-Widefield**[12]. Pseudo-Widefield averages images from raw SIM data and assembles, for comparison, an image equivalent to conventional widefield illumination[12]. For Image J itself see https://imagej.net/Welcome. Randomly select ~ 25 spores in each inverted transmission image for later germinosome analysis in the fluorescent Pseudo-Widefield images.

7.1.2. Select in total approximately 350 (in this example, 346) KGB80 spores in 14 fields of view from two slides. The GerD-GFP and GerKB-mCherry fluorescent foci numbers in selected KGB80 spores should be counted independently by two researchers. The researcher can refer back to the 3D-SIM raw images whenever in doubt of the presence of separate fluorescent germinosome foci in spores.

7.1.3. Assess the maximum intensities of each GerD-GFP and GerKB-mCherry focus and the integrated intensity of each KGB80 spore's 3D image with ImageJ.

7.2. Analyze Pseudo-Widefield images of the KGB80 germinosome

7.2.1. Use the mean integrated intensity value of 7 stacks as the integrated signal intensity of the KGB80 spore. Determine background intensities by imaging the background strain PS4150 using identical settings. Regard fluorescent spots in individual KGB80 spores as germinosome foci when they are clearly distinguishable from the background.

7.2.2. Apply one-way ANOVA-tests for significance determination with software Origin 9.0. considering P values <0.05 as statistically significant. Use the Spearman's rank correlation coefficient[18] to evaluate the correlation of GerD-GFP and GerKB-mCherry foci number and the measurements of the integrated signal intensity.

REPRESENTATIVE RESULTS:

The current protocol presents a SIM microscope imaging procedure for bacterial spores. The sporulation and slide preparation procedures were carried out as shown in **Figure 1** before imaging. Later, the imaging and analysis procedures were applied both for dim (fluorescent protein labeled germination proteins) and bright (lipophilic probe stained IM) spore samples as shown in the following text.

Localization of germinosomes in *B. subtilis* spores

Level of GerD and GerKB are reported to be ~3500 and ~700 molecules per spore, respectively, based on Western blot analyses of extracts from spores prepared in a rich sporulation medium[19]. Both the *gerD-gfp* and *gerKB-mCherry* genes in the KGB80 strain are under the control of their native promotor. The relative low abundance of fusion proteins led to a low fluorescent signal during imaging, so it was difficult to reconstruct such dim raw SIM images by the SIM reconstruction algorithm. However, the SIM microscope was still applied for the germinosome image acquisition, although the raw SIM images were converted into Pseudo–Widefield images by SIMcheck (ImageJ plugin). In addition, a seven stack 3D imaging was implemented to get a better overview of this IM focus. As shown in the left hand panel of **Figure 2**, two foci of GerD-GFP appeared in different stacks. The, in total, three GerD-GFP foci are indicated by the white arrows in the compositive column's Z3 stack. The right hand panel of **Figure 2** shows a spore with only one gerD-GDP focal point in the spore as evidenced by the white arrow in the composite column's Z4 stack. In total, around 40% and 50% of spores had two or one GerD-GFP and GerKB-mCherry cluster, respectively (**Table 1**). Among the 346 spores examined, two had 4 GerD-GFP foci, and one spore even had 5 GerD-GFP foci. Noticeably, in our hands, the number of GerD-GFP and GerKB-mCherry foci in the same spore were not always the same[18]. As the SIM microscope had no phase contrast option, we used transmission light microscopy for spore localization. Thus, spores appear with a dark and dense core surrounded by a brighter halo.

The integrated fluorescence intensity of KGB80 spores was measured by ImageJ. Spores, which had 0, 4, or 5 foci were not included into our statistical analysis due to their low frequency. There was a high positive correlation between GerD-GFP and GerKB-mCherry integrated intensities (Spearman rank correlation coefficient = 0.73). While the integrated intensity of the GerD-GFP scaffold protein was different between different populations (**Figure 3C**), the integrated intensity of GerKB-mCherry was about the same in different

populations (**Figure 3D**). The maximum fluorescence intensity of GerD-GFP and GerKB-mCherry foci tended to decrease, when the spore had multiple foci (**Figure 3A, B**). The maximum fluorescence of all bright spots, regarded as germinosome foci, was higher than the maximum auto-fluorescence of PS4150 spores (spores from the background strain KGB80; **Figure 3A, B**).

Organization of the inner membrane

As mentioned in the introduction, germinosome proteins are located in the spore's IM. However, few details are known about the biophysical properties of this largely immobile membrane. Exploring more details, such as the IM's local organization, would promote the understanding of the organization of IM proteins, in particular GRs and channel proteins. *B. subtilis* spores have a structure comprising multiple concentric layers, and a lipophilic probe cannot easily pass through these multiple layers to stain the IM surrounding the spore core. The passage of such probes is most likely hampered by the protein-rich coat layers and perhaps also the outer membrane[20,21]. To overcome this problem, the lipophilic membrane dye FM4-64 was added to a PS4150 culture during sporulation. By doing so, the PS4150 vegetative cell membrane was stained by FM4-64, and thus membranes in forespores obtained from this culture at the asymmetric sporulation cell division and subsequent forespore engulfment are well stained[5]. Consequently, the mature spore's membranes can be visualized. A previous study indicated that most if not all of the FM4-64 is in the IM in cleaned spores[5]. During an approximately 2 week period of incubation and spore purification treatments, the washing procedures applied remove any FM4-64 from the outer membrane, the latter effectively being removed following the decoating treatment and extensive washing steps[5]. However, the decoating procedure removes no FM4-64 from the IM, nor has any effect on the germinosome foci[5,6]. What excited us is that brighter FM4-64 spots similar to germinosome foci appeared in both intact (**Figure 4A, B**) and decoated spores (**Figure 4C, D**) of PS4150 spores. These brighter FM4-64 spots might be involved in the clustering of germinosome proteins in the IM.

FIGURE AND TABLE LEGENDS:

Figure 1: Overview of sporulation and slide preparation. An overview displaying the initial steps required before imaging. Detailed information is given in the protocol. (**A**) The schematic of the sporulation procedure in defined minimal MOPS medium. A *B. subtilis* PS4150 (PS832 Δ*gerE*::*spc* Δ*cotE*::*tet*) or KGB80 (PS4150 *gerKA gerKC gerKB-mCherry cat gerD-gfp kan*) single colony was cultured in 5 mL of LB rich medium, and adapted in 5 mL and 20 mL of MOPS medium in turn, and finally sporulated in 250 mL of MOPS medium. An early exponential phase culture (OD_{600}, 0.3-0.4) is used in all intermediate cultures. FM4-64 (2 µg/mL) was added to the PS4150 sporulation medium for spore membrane staining 1 or 2 h after reaching the peak OD_{600} value. (**B**) Method of harvest spores from MOPS sporulation culture and purify spores by density gradient centrifugation. (**C**) Procedure of stabilizing spores on 1 % agarose pad in a gene frame chamber.

Figure 2: Representative Pseudo-Widefield (PWF) 3D images of GerD-GFP and GerKB-mCherry foci in two KGB80 (PS4150 *gerKA gerKC gerKB-mCherry cat gerD-gfp kan*) dormant spores' 3D-SIM raw images were taken with dual channel excitation (561nm, 30% laser power, 1 s, and 488nm, 60% laser power, 3 s) using 7 steps from top to bottom Z-stacks. Subsequently, the raw SIM images were converted into Pseudo-Widefield 3D images

by the ImageJ plugin SIMcheck. From left to right, 3D images (Z2-Z5) of GerD-GFP (green), GerKB-mCherry (red) and the corresponding composite images of two KGB80 spores (i and ii) are shown in the panel. Transmission (Trans) light images of two spores (i and ii) indicated the location of spores that appear as dark dense images surrounded by a brighter halo. The scale bar is 1 µm and all panels are at the same magnification.

Figure 3: The maximum fluorescence intensity of GerD-GFP in KGB80 (PS4150 *gerKA gerKC gerKB-mCherry cat gerD-gfp kan*) dormant spores, and the maximum auto-fluorescence intensity of PS4150 spores in arbitrary units. All spores were illuminated by the settings indicated the protocol. Panels (**A**) and (**B**) show the maximum fluorescence intensity of the GerD-GFP and GerKB-mCherry foci, respectively, in KGB80 dormant spores as well as in both cases the maximum auto-fluorescence intensity of the parent PS4150 spores. Panels (**C**) and (**D**) show the integrated fluorescence intensity of the GerD-GFP foci and the integrated fluorescence intensity of the GerKB-mCherry foci, respectively, in *B. subtilis* KGB80 dormant spores. We used one-way ANOVA-tests for significance determination of differences in maximum focal point intensity and integrated spore fluorescence intensities with software Origin 9.0 considering P values <0.05 as significant. Spores with 4 or 5 foci were excluded from the analysis because of their low abundance. The data is represented in notched boxplots. The notches in the plots are around the median values observed with their width proportional to the interquartile range (IQR). The whiskers shown represent a maximum of 1.5 the IQR. Asterisks indicate a significant difference of median values. GerD-GFP and GerKB-mCherry integrated fluorescence intensities have a strong positive correlation (Spearman correlation coefficient = 0.73)[18].

Figure 4: Representative Pseudo-Widefield (PWF) images (A and C) and reconstructed SIM images (B and D) of the FM4-64 stained IM of *B. subtilis* PS4150 (PS832 *ΔgerE::spc, ΔcotE::tet*) spores. FM-464 was incorporated into spores during sporulation. 3D-SIM raw images of intact spores (**A** and **B**) and decoated spores (**C** and **D**) were taken with one channel excitation (561 nm laser, 20% laser power, 400 ms) using a 25 step top to bottom Z-stack. Subsequently, the raw SIM data was reconstructed by the microscope imaging software (see **Table of Materials**) into 3D-SIM images, or converted into PWF by SIMcheck (ImageJ plugin). The cyan arrows point to FM4-64 foci in the IM in panel **B** and **D**. The scale bar is 1 µm and all panels are at the same magnification.

Table 1: Presence of foci in KGB80 spores. The germinosome foci number per spore in a population of dual labeled *B. subtilis* KGB80 (PS4150 *gerKA gerKC gerKB-mCherry cat gerD-gfp kan*) dormant spores. Fluorescence in spores was counted as germinosome foci when a focus' maximum intensity was higher than the auto-fluorescence intensity, which was the maximum intensity of PS4150 (PS832 Δ*gerE*::*spc* Δ*cotE*::*tet*) dormant spores excited by the same illumination settings as the KGB80 spores.

DISCUSSION:

The protocol presented contains a standard 3D-SIM procedure for analysis of FM4-64 stained *B. subtilis* spores that includes sporulation, slide preparation and imaging processes. In addition, the protocol describes a modified SIMcheck (ImageJ)-assisted 3D imaging process for SIM microscopy of *B. subtilis* spore germinosomes labeled with fluorescent reporters. The latter procedure allowed us to observe this dim substructure with enhanced

contrast. By coupling two imaging procedures, it is possible to visualize discrete sub-structures in the same spore with the same SIM microscope, thus improving our basis for a mechanistic understanding of the germination process. Note that the procedure operates at a lateral resolution of ~100 nm and an axial resolution of ~200-250 nm. This is better than the Differential Interference Contrast (DIC) wide-field microscopy approach used by Griffith[7]. Time resolved analysis of germinosome appearance upon initiation of germination would be a desired next step. Unfortunately, though SIM microscopy is in principle compatible with live-imaging, due to the dim nature of the germinosome signals such time-resolved SIM, analyses are not feasible because of rapid bleaching of the samples during image acquisition. In order to obtain sufficient spores for analysis, it is crucial to make sure that sporulation is efficiently taking place. Researchers must therefore check sporulation efficiency meticulously with 90% efficiency as the target. In the representative results, in dormant spores, respectively ~ 50% and 40% of all spores have one or two GerD-GFP and GerKB-mCherry foci (**Table 1**). The percentage of spores with two foci is much higher than that reported by Griffiths previously[7]. There are several reasons that could explain the different result in the current work. First, the 3D imaging process could facilitate the detection of more foci. Different foci in the same spore are located in different locations in the vertical direction as shown in **Figure 1**. Second, the CCD camera (**Table of Materials**) and laser unity equipped to the SIM microscope contribute significantly to the imaging results. Third, similar to Griffiths's approach[7] to average dozens of consecutive images for better image analysis, the Pseudo-Widefield image of the germinosome was also an average image from raw SIM images (5 phases and 3 orientations images). Finally, the sporulation medium and sporulation conditions, an important variable in determining spore properties, are different in our work from that used previously. Griffiths et al.[7] used rich 2x Schaeffer's-glucose (2x SG) medium for sporulation, while a defined minimal MOPS buffered medium was employed here. Several papers have demonstrated that sporulation medium and conditions have significant effects on the protein composition, resistance, and germination of *B. subtilis* spores[22-25]. Indeed, it has been shown that levels of GR subunits are 3- to 8-fold lower in spores obtained on a poor medium versus those obtained on rich-medium. GerD levels were also around 3.5-fold lower in poor medium spores, and these spores took longer to start spore germination[26]. However, it is not clear whether sporulation conditions also influence the number of observed germinosome foci.

Ramirez-Peralta et al.'s results[26] indicated that rates of nutrient germination of spores at population levels are influenced significantly by the levels of germination proteins and GerD. If the integrated fluorescent intensities per spore from the fluorescent reporters are directly proportional to the levels of GerD and GerKB fusion proteins, levels of both fusion proteins differ widely in KGB80 spores, which is in agreement with previous work[7]. This protein level heterogeneity might be related to spore germination heterogeneity observed at the single spore level, and germinosome foci number might be another factor contributing to spore germination heterogeneity. Further experiments will focus on an analysis of the possible effect that germinosome foci number and foci germination protein composition (not all germinosomes may be equal in germination protein composition) could have on germination heterogeneity. The data gave rise to a number of current research questions including: i) what is the role of GerD in the clustering of GRs in the IM; and ii) how are the two other GRs, GerA and GerB, organized in the spore IM?

The protocol presented for dim and bright spore samples makes it possible to visualize discrete sub-structures in the same spore by SIM microscopy. The bright FM4-64 spots that were observed in spores might be due to extensive folding of the IM[27]. Alternatively, we hypothesize that these regions are areas of the IM where the dye could more easily gain access to due to increased local IM fluidity. Such Regions of Increased Fluidity (RIFs) may be organized by the cytoskeletal actin homologue MreB, well known for its concentration of fluid short acyl chain lipids[28,29]. Noticeably, applying the same procedure to wild-type *B. subtilis* spores also leads to a similar pattern of bright FM4-64 spots (our unpublished observations). In *B. subtilis* vegetative cells, a collapsed membrane potential results in the clustering of MreB and RIFs[29]. The inner membrane of dormant spores likely has a relative low membrane potential[20,21] and contains detectable levels of MreB[30] which might lead to the clustering of RIFs into larger domains of high fluidity[29]. Whether such domains could coincide with the presence of germinosomes is currently under investigation.

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DISCLOSURES:

No conflicts of interest declared.

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Figure 1

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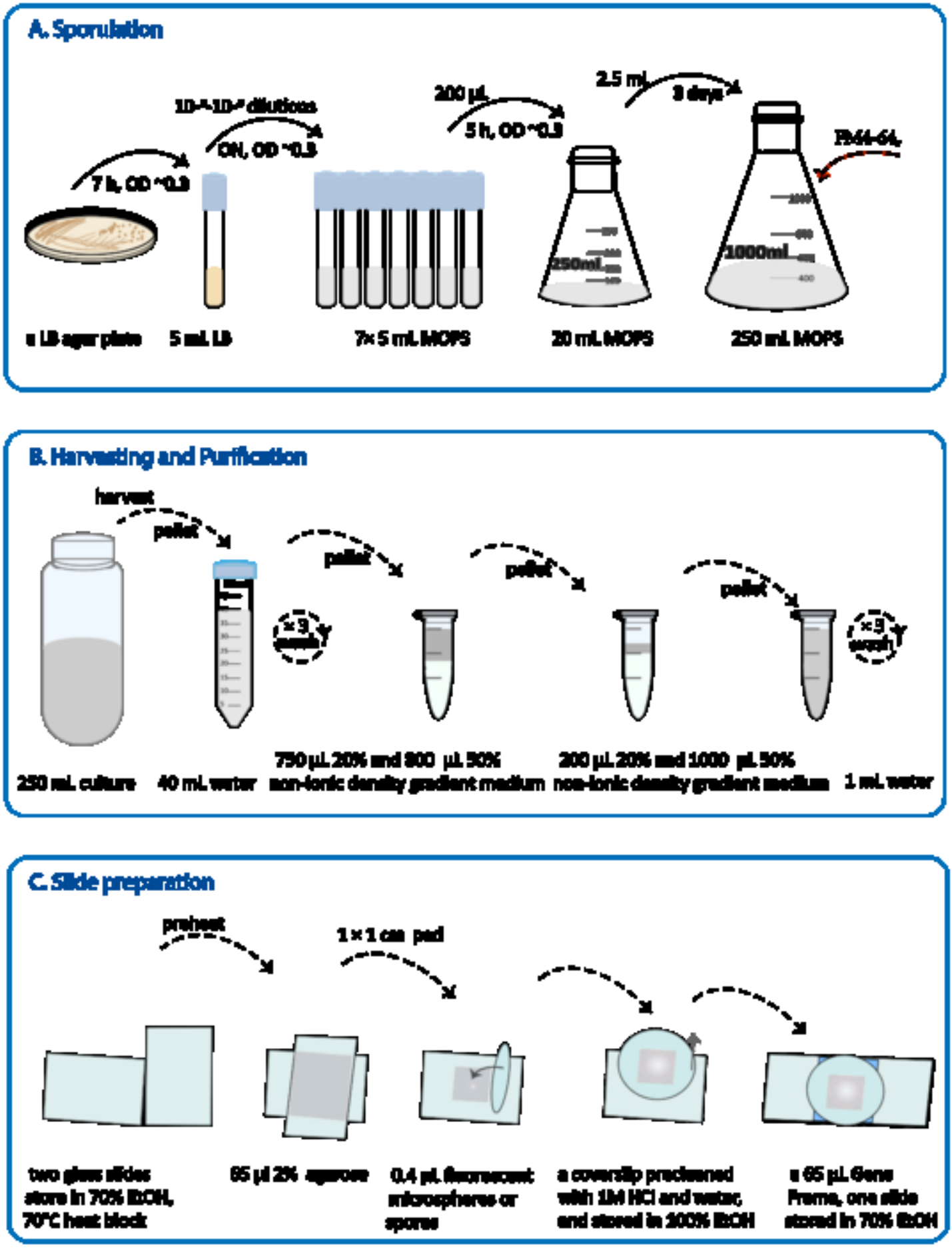

Figure 2

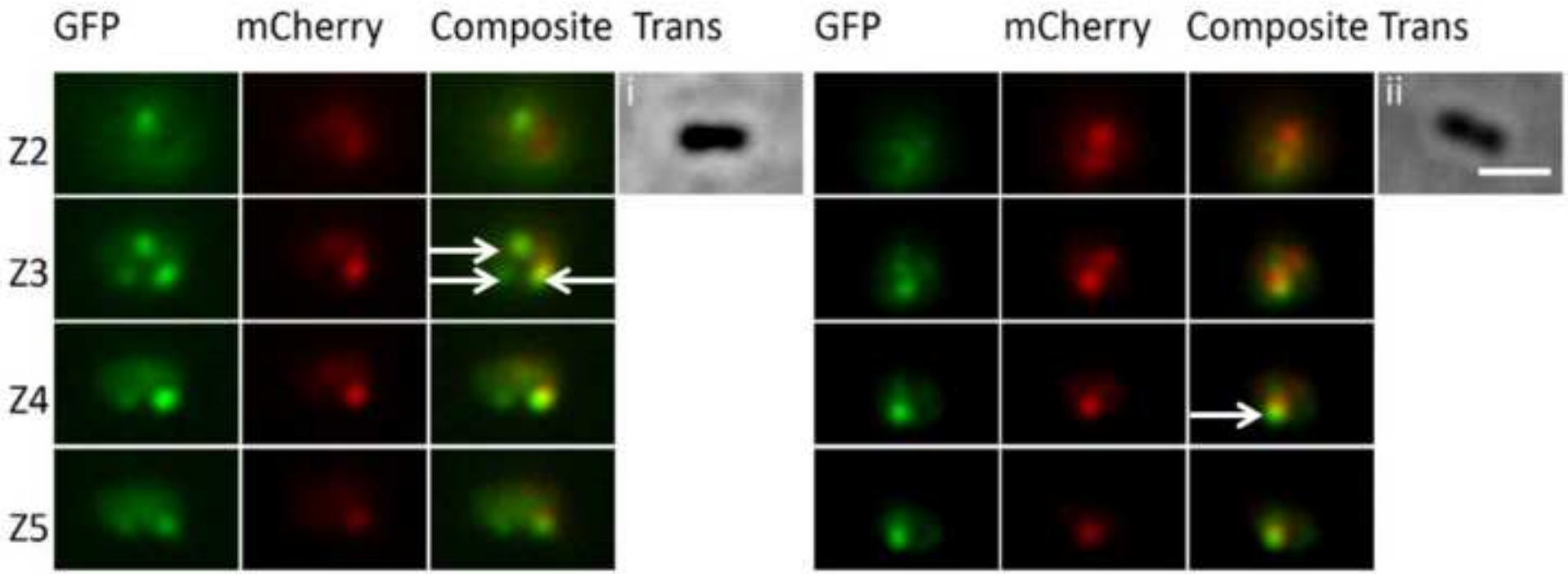

Figure 3

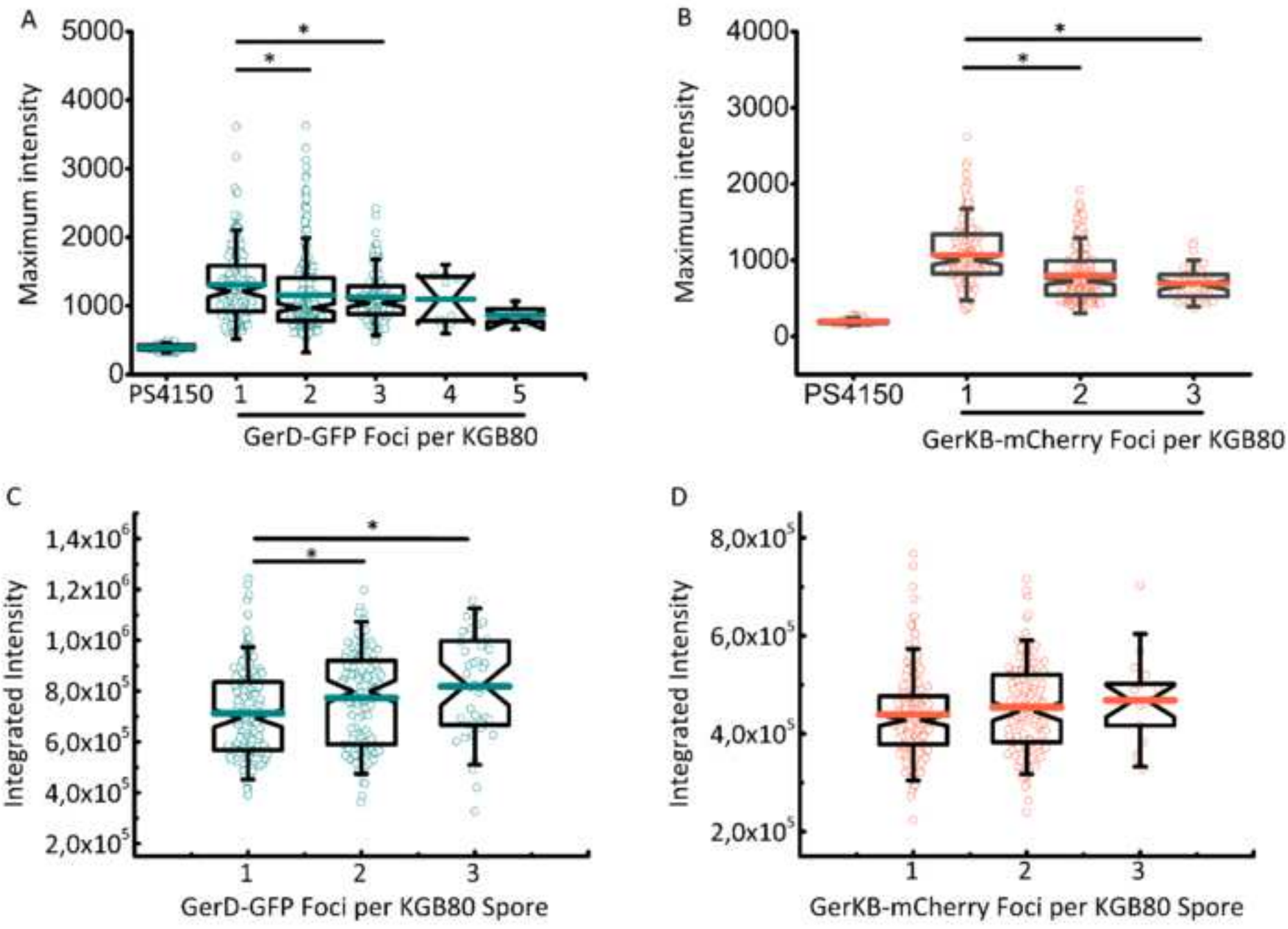

Figure 4

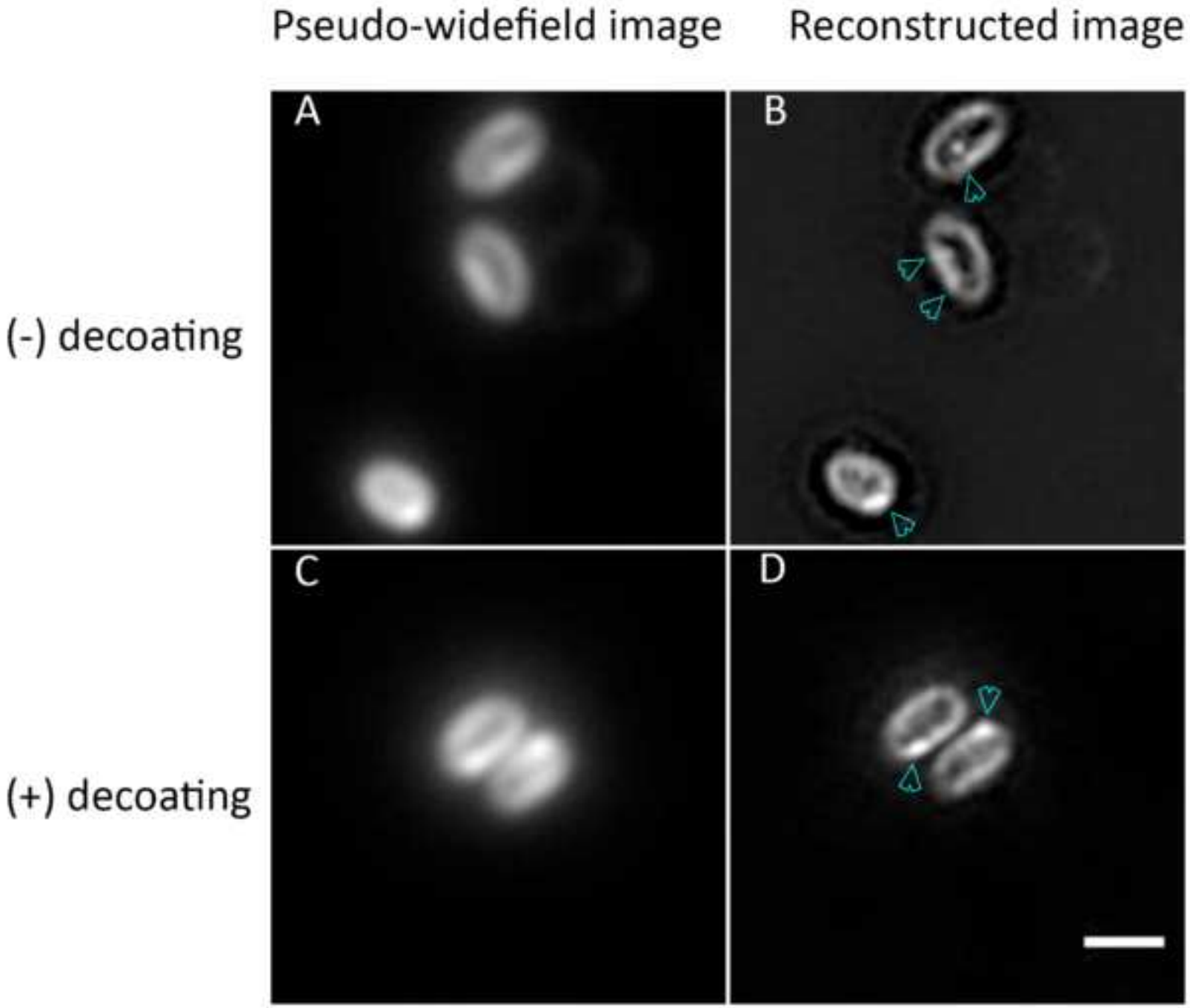

Table 1. Presence of foci in KGB80 spores

Strain	Spores counted	Foci	Foci per spore (%)			
			0	1	2	3
KGB80	346	GerD-GFP	2	46	40	11
		GerKB-mCherry	2	53	40	5

4	5
1	0
0	0

Table of Materials

Click here to access/download;Table of Materials;Wenetal2018JoVE_Materialsupdated.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Air dried glass slides	Menzel Gläser	630-2870	
APO TIRF N20R8 100× oil objective (NA=1.49)			
B. subtilis KGB80 (PS4150 *gerKA gerKC gerKB-mCherry cat, gerD-gfp kan*)			
B. subtilis PS4150 (PS832 *ΔgerE::spc, ΔcotE::tet*)			
Erlenmeyer flasks 1 L	Sigma-Aldrich	Z567868	
Erlenmeyer flasks 250 mL	Sigma-Aldrich	Z723088	
FluoSpheres carboxylate-modified microspheres	Invitrogen, Thermo Fisher Scientific	F8803	
FM4-64		F34653	
Histodenz nonionic density gradient medium	Sigma-Aldrich	D2158	
Image J			
iXON3 DU-897 X-6515 CCD camera	Andor Technology		https://imagej.net/Welcome
LB Agar	Sigma-Aldrich	L2897	
Microfuge tubes 1.5 mL	Thermo Fisher Scientific	3451PK	
Microscope imaging software	Nikon, Japan	NIS-Element AR 4.51.01	
MilliQ Ultrapure Deminerilzed Water	Millipore	Milli-Q IQ 7003	
Nikon Eclipse Ti microscope			

Polypropylene Screw Cap Bottle 18	Thermo Fisher Scientific	75003800	
Precision Coverslips	Paul Marienfeld	117650	
Round Bottom tubes 15 mL	Thermo Fisher Scientific		Nunc TM
Screw cap tubes 50 mL	Thermo Fisher Scientific		Nunc TM

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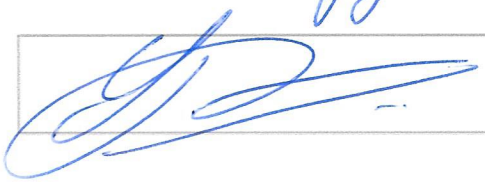
Signature: 

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January 12th 2019

Concerns rebuttal of the manuscript **'Visualization of the germinosome and the inner membrane in *B. subtilis* spores'**

Dear editor,

Hereby we would like to submit our revisions of the manuscript,

'Visualization of the germinosome and the inner membrane in *B. subtilis* spores'

by Juan Wen, Raymond Pasman, Erik M.M. Manders, Peter Setlow and Stanley Brul

Below we answer point by point the editorial comments made with reference to version 59388_R1. Please note that in the revised version we extended the yellow highlighted part of the protocol text to section 7. We realised this omission and truly hope that it will be possible to include also this part of the protocol in the video.

Editorial comments:
Changes to be made by the author(s) regarding the manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Author's answer: This has been done to the best of our abilities.

2. The Short abstract is over the 50 word limit.

Author's answer: The short abstract has been rewritten and is now 46 words.

3. Please use h, min, s for time units.

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4. For steps that are done using software, a step-wise description of software usage must be included in the step. Please mention what button is clicked on in the software, or which menu items need to be selected to perform the step.

Author's answer: This has been changed in the revised text.

5. Step 1.1.1: Please ensure that all text is written in the imperative tense.

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7. Figure 3: Please add a title for the whole figure.

Author's answer: The legend of the figure has been modified accordingly.

We hope that with these answers to the editorial points raised the manuscript is now acceptable for publication in the Journal of Visualised Experiments.

Yours sincerely,

Stanley Brul

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