Fluorescence Assay for Spore Germination Detection

Fast Decontamination Assay

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***Abstract*—Tracking viability of spores for assessing decontamination and sterilization quality is of utmost importance in medical and other clean environments. However, classical tests for spore contamination rely on bacterial growth, which is slow. Rather than monitoring bacterial growth, one can detect the spore germination process. Here, we present our method for reliable testing for spore germination by fluorescence detection of the associated DPA release. Extrapolating our results, we estimate that single spore detection will be possible with our method.**

Keywords – germination; time resolved fluorescence, component; spore detection; sterilization detection.

I. INTRODUCTION

Tracking viability of spores for assessing disinfection and sterilization quality is of uttermost importance in medical and other clean environments. Biological indicators, or spore tests, are the most accepted means to assess the sterilization process. They directly control the sterilization by exposing known highly resistant microorganisms and then controlling for residual bacterial growth, which is a slow process extending over several days [1].

Rather than monitoring bacterial growth, one could detect the germination process. We are developing a method for reliable testing for spore germination. During spore germination, dipicolinic acid (DPA) is released [2], which can be monitored by adding Tb^{3+}-ions to the solution and monitoring the fluorescence [3].

Here, we present details on our specific approach and first experimental results. Extrapolating our results to microfluidic systems, we estimate that single spore detection will be possible. In Section II the experiment is described and in Section III the results are presented and discussed.

II. EXPERIMENTAL

The measurement consists in the determination of the fluorescence from a Tb^{3+} complex formed with DPA. Terbium (Tb^{3+}) is chosen as it forms a complex with high fluorescence quantum yield [4].

For the investigated use case, we developed a laboratory setup based on time-resolved fluorescence collection as schematically shown in the inset of Figure 1. Briefly, UV pulses at 266 nm with a repetition rate of 18 Hz were used to excite the sample. The emitted fluorescence was collected orthogonally to the excitation by a large numerical aperture lens and refocused onto a Silicon photomultiplier (SiPM) with a 3x3 mm^2 active area (Ketek, PM3315-WB). No spectral filter was used in the detection path, to maximize the fluorescence collection. Instead, the signal was temporally resolved, which allows to filter out short-lived contributions from autofluorescence or scattered light.

Figure 1. shows fluorescence measurements for different concentrations of DPA. Signals start to saturate at ~500 nM. Concentrations below 50 nM can still be well quantified. These curves were used for calibration, in order to estimate DPA concentrations in the samples.

From different tests, the best conditions had to be determined, in order to provide favorable biochemical conditions for spore germination, which, on the other hand do not interfere with the fluorescence measurements. The indicator spores we used were *Geobacillus stearothermophilus* obtained from Merck.

The best results were achieved using 10 mM Trizma buffer, in part because its pH can be tuned to the value of 8.0, a commonly accepted optimum for the germination of spores of *Geobacillus stearothermophilus.*

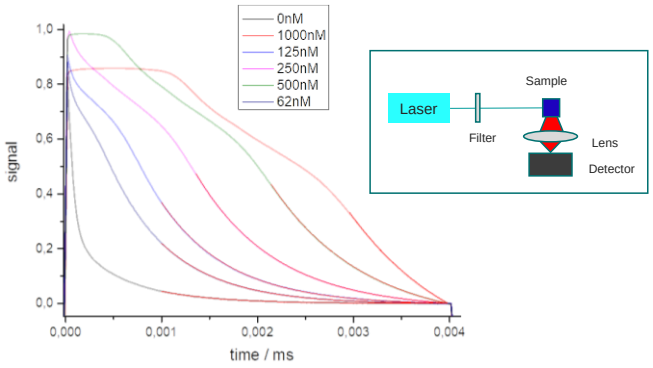

Figure 1. a) Fluorescence intensity of TbDPA in 10mM Trizma buffer for the DPA concentrations marked in the legend; Curves at high concentrations show detector saturation. (Inset shows a scheme of the fluorescence set-up)

Geobacillus stearothermophilus typically requires a relatively complex nutrient mixture for efficient growth [5]. Nevertheless, it was demonstrated that the early steps in the transition from a spore to a vegetative cell can be induced in simple media containing organic acids [6]. Here, incubation

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was conducted in a solution containing 1 mM L-valine solution at 65°C. Sterilization was done by exposure of the spore-samples to H_2O_2 over 45 minutes.

The overall measurement process is shown in Figure 2.

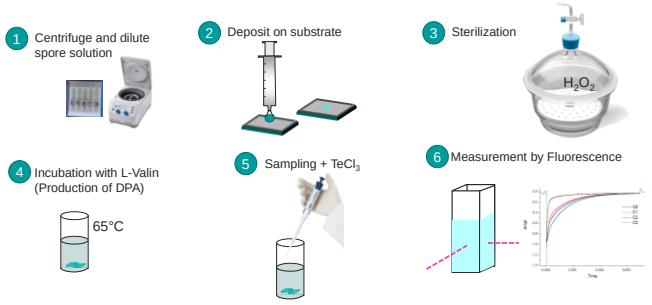

Figure 2. Process flow, as described in text

A spore solution (*Bacillus stearothermophilus*, Merck) was centrifuged, in order to remove residual DPA in the solution and then deposited on a substrate, which was exposed to H_2O_2 vapor for approximately one hour for sterilization. Subsequently the sample was dissolved in buffer solution (Tris, pH=8) and L-Valin was added to support germination. Germination was triggered by heating to 65°C and samples were taken after 30, 90, and 120 minutes of incubation. Flourescence decays were measured after adding $TbCl_3$.

III. Results and Discussion

Results showing the kinetics of germination are summarized in Figure 3. Spore solutions with 10^7, 10^6 and 10^5 CFT/ml were prepared and centrifuged for removing DPA from the solution. Germination was initiated by adding L-valine and heating to 65°C. Samples were taken at 0, 30, 90, and 120 minutes.

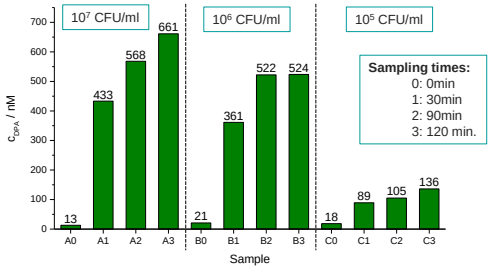

Figure 3. DPA-concentration measured on the germination samples. Sampling times are indicated in the inset. Concentrations of the original spore solutions were 10^7, 10^6, 10^5 CFT/ml for sample-series A, B, C, respectively. (Concentrations were estimated by comparing the measured fluorescence decays with calibrated samples like in Figure 1)

Estimating an average DPA content of 2.3 10^8 molecules per spore and assuming a fraction of 3% of DPA released by the spores [6], we estimate the concentration of DPA after germination of the spore suspension above (10^6 CFU/ml) of about 11 nM. Indeed, the jump in DPA concentration from germination can easily be measured and it is in the range of expected signal.

Extrapolating to the single spore regime, we would obtain a similar DPA concentration by confining the volume to the nanoliter. One spore confined to a single nl, would release a DPA amount of 12 attomol corresponding to an effective concentration of 11 nM within the small volume. Thus, single spore measurements are attainable in a microfluidic environement, where the spore and laser light are confined to a volume of 1 nanolitre, corresponding to channel dimensions in the order of 100 µm. This should be achievable in a micro-fluidic set-up combined with microscopic fluorescence detection. Further experiments will be necessary to get a better estimate about repeatability and measurement errors.

IV. Conclusions

Overall, we present first results from our approach of a rapid assay for spore germination detection, which can be used for sterilization validation. Using an optimized combination of germination conditions and a highly sensitive time-resolved fluorescence detection set-up based on pulsed laser excitation, we could determine the DPA, which is released during germination with nM sensitivity under realistic conditions.

We also estimated that single spore detection could be realistic, however, in order to reach sensitivities approaching the single spore regime, a microfluidic environment would be necessary.

Acknowledgment

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