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Differentiating between live and dead *Mycobacterium smegmatis* using autofluorescence

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Abstract

While there have been research efforts to find faster and more efficient diagnostic techniques for tuberculosis (TB), it is equally important to monitor a patient's response to treatment over time, especially with the increasing prevalence of multi-drug resistant (MDR) and extensively-drug resistant (XDR) TB. Between sputum smear microscopy, culture, and GeneXpert, only culture can verify viability of mycobacteria. However, it may take up to six weeks to grow *Mycobacterium tuberculosis (Mtb)*, during which time the patient may have responded to treatment or the mycobacteria are still viable because the patient has MDR or XDR TB. In both situations, treatment incurs increased patient costs and makes them more susceptible to host-drug effects such as liver damage. Coenzyme Factor 420 (F_{420}) is a fluorescent coenzyme found naturally in mycobacteria, with an excitation peak around 420 nm and an emission peak around 470 nm. Using *Mycobacterium smegmatis*, we show that live and dead mycobacteria undergo different rates of photobleaching over a period of 2 min. These preliminary experiments suggest that the different photobleaching rates could be used to help monitor a patient's response to TB treatment. In future studies, we propose to describe these experiments with *Mtb* as both *M. smegmatis* and *Mtb* use F_{420}.

Keywords

Mycobacterium; Autofluorescence; Viability; Microscopy

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Conflict of interest

none.

1. Introduction

Tuberculosis (TB) is an infectious disease caused by the bacterium *Mycobacterium tuberculosis* (*Mtb*) and is the leading killer worldwide due to a single infectious agent according to the World Health Organization (WHO). Like many diseases, TB is curable and preventable but only if a patient is properly diagnosed and treated [1]. While there have been research efforts to find faster and more efficient diagnostic techniques, it is equally important to monitor a patient's response to treatment over time, especially with the rising number of multi-drug resistant (MDR) and extensively-drug resistant (XDR) TB cases.

There are three main methods for TB diagnosis: sputum smear microscopy, bacterial culturing techniques, and GeneXpert. Historically, sputum smear microscopy is the oldest diagnostic method for mycobacteria with the invention of the acid fast bacilli (AFB) staining with the Ziehl-Neelsen (ZN) stain in 1882 [2]. ZN is the most frequently used AFB staining method and it is simple to implement, cost-effective, and a rapid test for TB. However, sputum smear microscopy suffers from low sensitivity (ranging from 20 to 90%) due to user dependent diagnoses and the pool population [3]. Mycobacterial culturing is the current gold standard for TB diagnostics. The sensitivity of culture is generally higher and more precise than that of smear microscopy, ranging from 80 to 85% with specificity around 98% [4]. It can, however, take up to six weeks to diagnose a patient due to the slow dividing time of *Mtb*, though this can be shortened with different growth media. In the past few years, GeneXpert successfully introduced to the market polymerase chain reaction (PCR) based methods for TB diagnosis. GeneXpert's sensitivity and specificity are close to that of culturing with the added benefit of reduced diagnosis time, usually measured in hours as opposed to days or weeks in the case of culturing. While perfectly acceptable in controlled laboratory environments, GeneXpert faces difficulties in the field due to the requirement of a constant, uninterrupted energy supply and sensitivity to microbiologic contamination in both sample collection and pre-processing stages. It is expected that technical infrastructural requirements will be lowered with the introduction of the small, portable GeneXpert Omni, which is expected to be available at the point-of-care in late 2017 [5].

After a patient is diagnosed with TB and begins treatment, monitoring to determine treatment responsiveness is critical. Monitoring may be done by performing additional sputum smears or GeneXpert assays, or by culturing for *Mtb*. However, sputum smears only look for the presence of AFB and GeneXpert only assays for *Mtb* DNA; thus, it is possible to have smear or GeneXpert positive results but have a culture negative result as all the mycobacteria may be dead and have not cleared the system. Culturing techniques can be used to test for viability, but as mentioned culturing may take up to six weeks to grow the mycobacteria, during which time the patient may have already responded to treatment and is no longer infectious. In all cases, treatment may take longer than necessary for a patient, increasing costs for both the patient and the hospital (in the use of negative pressure isolation rooms in the developed world). Conversely, it may be possible that the patient is not responding to their treatment because they have MDR or XDR TB, in which case the patient spent a significant amount of time taking an ineffective, toxic drug (usually rifampin) [6]. This again prolongs the patient's treatment time as the patient may need to transition to more

potent drug regimens thus making the patient more susceptible to host-drug effects such as liver damage.

Coenzyme Factor 420 (F_{420}) is a two factor transfer coenzyme that is found primarily in prokaryotes such as cyanobacteria and methanobacteria. Recently, it was discovered that *Mycobacterium* and *Nocardia* also share this coenzyme, though its role within *Mycobacterium* has not been fully explored [7,8]. Because of its fluorescent property, F_{420} is thought to be the main factor in the intrinsic fluorescence (or autofluorescence) for these microorganisms. The F_{420} coenzyme is naturally present in *Mtb* and in *Mycobacterium smegmatis (M. smegmatis)* and has an excitation peak centered around a wavelength of 420 nm and an emission peak centered around a wavelength of 470 nm [9].

Like other fluorescent materials, F_{420} may photobleach over time. In experiments by Schneckenburger et al., it was discovered that *Methanobacterium* (which uses F_{420}) exhibited different photobleaching behaviors based on whether the methanobacteria were active or inactive [10]. The active methanobacteria photobleached at a slower rate than that of inactive methanobacteria, thus authors were able to distinguish between active and inactive organisms by measuring their respective photobleaching rates.

We hypothesize that live and dead *Mycobacterium* should exhibit similar behavior to that of the *Methanobacterium*, in that the live mycobacteria should exhibit a different photobleaching rate than that of the dead mycobacteria. For this proof of concept project, we focused on *M. smegmatis*. This allowed us to perform all experiments in a biosafety level (BSL) 2 laboratory. In future studies we hope to show similar results for *Mtb* microorganisms performed in a BSL 3 laboratory, as both *M. smegmatis* and *Mtb* utilize F_{420}.

2. Methods

2.1. Sample preparation

M. smegmatis (ATCC 21732) was subcultured in Middelbrook 7H9 broth (Fischer, BD part number 221832) and Tween 80 (Fischer, BP338-500). They were then grown in a 37 °C CO_2 incubator for a week and extracted during the log phase of their growth. The mycobacteria were then separated into three different groups: live mycobacteria, mycobacteria killed with 70% ethanol, and mycobacteria killed with BD Cytofix/Cytoperm™ Fixation/ Permeabilization (BD Perm/Fix; San Jose, California). Each mycobacterial variation was prepared to an optical density of 1, which is approximately 3×10^8 cfu/ml.

A 10 μl inoculating loop was used to plate a sample of each variation of the mycobacteria on BD BBL Stacker plates (A7 agar; San Jose, California). Two plates were made for each variation. A sample of 20 ml was extracted with a variable pipette and placed on a microscope slide. The slide was then covered with a coverslip and sealed with clear nail polish. Five microscope slides were prepared for live mycobacteria, mycobacteria killed with 70% ethanol, and mycobacteria killed with BD Perm/Fix (totaling 15 sample slides for each experiment).

2.2. Imaging setup and image processing

For each of the 15 samples, the sample was placed on a Zeiss Axio Imager Z1 microscope (Oberkochen, Germany) with a 40x, 0.65NA objective. Each sample was brought into focus in brightfield mode and a brightfield image was taken with an AxioCam MRc5 camera (Oberkochen, Germany) at a 150 ms exposure time. The microscope was placed into fluorescence mode with an excitation filter at 390 nm (Semrock part number FF01-390), an emission filter at 460 nm (Semrock part number FF01-460), and a dichroic mirror (Semrock part number FF416-Di01-25×36). Images were taken over the course of a 2 min period in 10 s intervals.

Images were post-processed to visualize the autofluorescence intensity trend over time. For each sample, the average intensity value (AIV) was calculated for each of the thirteen images starting from 0 s to 120 s. These values were then normalized by the initial AIV (corresponding to the time = 0 s image). In normalizing the AIV, we account for the variability in intensity due to the different size of the mycobacterial clusters between samples. The normalized AIV were then plotted to visualize the intensity changes over time for each sample in a particular experiment. A final plot was created to show the average normalized intensity for each time point over all experiments, with standard deviation error bars around each time point.

3. Results and discussion

Fig. 1 shows an example brightfield image of a microscope slide with *M. smegmatis* that had been killed with BD Perm/Fix. The large dark yellow region is the main clump of mycobacteria, while smaller nodules in the background are likely smaller clumps of mycobacteria. Fig. 2 shows the corresponding time lapse images taken with the fluorescence mode of the microscope, starting from time 0 s in the upper left corner to time 120 s in the last image and separated by 10 s each.

The results from the experiments are presented in Fig. 3. The green lines represent the normalized AIV of the live mycobacteria, the blue lines represent the normalized AIV of the mycobacteria killed with 70% ethanol, and the red lines represent the normalized AIV of the mycobacteria killed with BD Perm/Fix. Each line represents a different sample.

Over the course of 2 min, mycobacteria killed with 70% ethanol and mycobacteria killed with BD Perm/Fix showed a decreasing intensity trend starting from the initial time point. In contrast, the live mycobacteria generally showed an increasing intensity trend approximately within the first 30 s before decreasing in intensity. Combining the data from the three experiments, each time point in Fig. 4 is the average normalized AIV for that particular time point with standard error bars (representing one standard deviation) around that particular time point.

While the mycobacteria that were killed with either 70% ethanol or BD Perm/Fix behaved as expected with a decaying intensity curve, the live mycobacteria increased in intensity within approximately the first 30 s before finally beginning to decay. In the future, this may be used as an indication whether the mycobacteria are alive or are dead, as all the mycobacteria that

had been killed immediately began dropping in intensity from the initial time point, while the live mycobacteria immediately began to increase in intensity from the initial time point. As these experiments dealt with samples of either all-live or all-dead mycobacteria, additional experiments will be performed to measure how sensitive this method is to different ratios of live:dead populations.

A limitation for our method is both latent TB infection and non-replicating persistence (NRP) of *Mtb*. Data from both macrophage physiology and the nature of tuberculous lesions in man and animals suggests that hypoxia is a major factor in inducing NRP of *Mtb* [11]. Conversely, it has been shown that activated macrophage produce NO, which in acidified phagosomes is converted to NO_2. Thus by converting NO_2 back to NO with $F_{420}H_2$, *Mtb* may also decrease the effectiveness of antibacterial action of macrophages; such a defense would correspond to active tuberculosis conditions where the bacterium grows aerobically [12]. Epidemiologically, public health workers and clinicians are worried about TB transmission, which only occurs when the patient has active disease state where growth (and thus *Mtb* metabolism) must occur. Therefore, though our method would likely be unable to detect latent TB infection or NRP of *Mtb*, we should be able to detect actively growing and metabolizing *Mtb*.

While a short exposure time works well for tightly clumped areas of mycobacteria, exposure times do not necessarily need to be the same for all experiments since the data was normalized to the initial image intensity value and exposure time is a linear relationship. In future experiments, different exposure times may be useful in capturing the fluorescence of sparse mycobacteria in samples.

Although we normalized the AIV of each image to account for different 2-dimensional areas of mycobacteria between images, we did not take into account the thickness of the cluster of mycobacteria (3-dimensional data). Post-processing procedures will need to be refined to account for the thickness of the mycobacterial cluster to normalize between samples. In the future, this may be done by additional normalization steps that take into account the intensity of the mycobacteria in the brightfield image, as thicker samples would most likely appear darker. Additionally, BD Perm/Fix is normally used for killing *Mtb*, and is not a standard method for fixing *M. smegmatis*. While initial tests suggest that BD Perm/Fix is an efficient permeabilization agent, further validations will be necessary to show that BD Perm/Fix is an effective fixation/permeabilization agent against *M. smegmatis.*

4. Conclusion

After starting a treatment regimen, TB patients may need to undergo additional tests to monitor their response to a treatment regimen. With sputum smear microscopy and GeneXpert, one cannot determine whether mycobacteria within a patient are alive or dead, as both methods only determine the existence of either acid fast mycobacteria or DNA respectively. This may prolong the time that the patients must remain in the hospital, either in a negative pressure room for developed countries (which may cost around $15,000 per day to utilize [13] though this number is highly variable) or isolated in other process, such as

cohorting. This may also prolong the treatment for the patients, leading to increased risk of host-drug effects such as liver damage.

Live mycobacteria appear to show an increasing intensity trend within the first 30 s, while both methods for dead mycobacteria appear to show a continuously decreasing trend over time. In the future, F_{420} autofluorescent intensity measurements may be used as a rapid test for viability of mycobacteria. Further experiments need to be performed with *Mtb* to ensure the results will translate from the results seen with *M. smegmatis,* to test the sensitivity of our method on mixed live and dead populations, to account for the differing thicknesses of mycobacterial clusters between samples, and to validate that BD Perm/Fix is an effective method for killing M. smegmatis.

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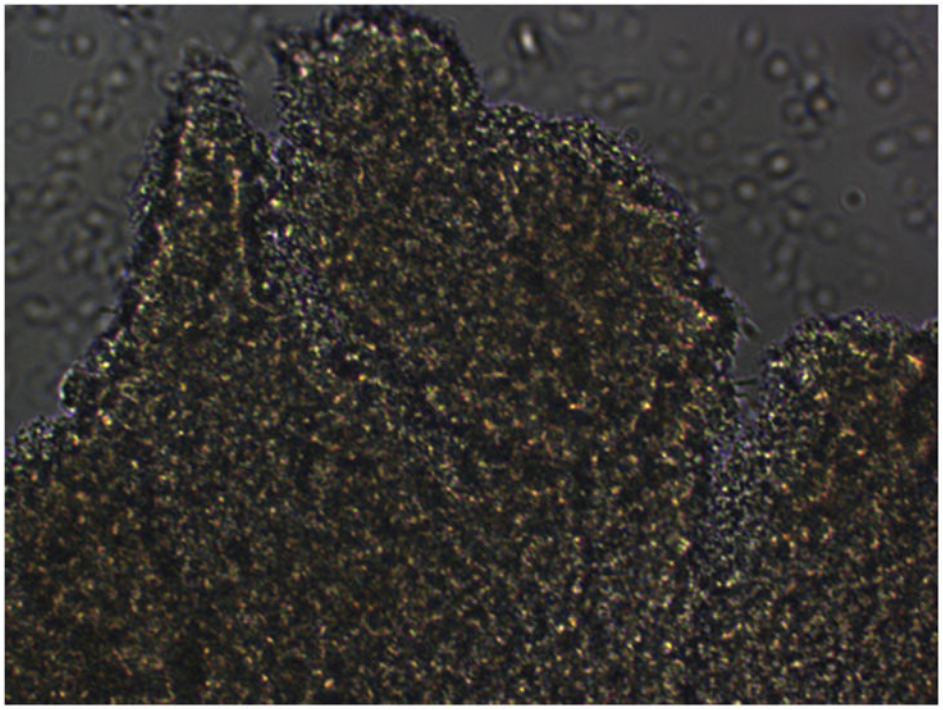

Fig. 1.
Brightfield image of a microscope slide with mycobacteria that have been killed with BD Perm/Fix.

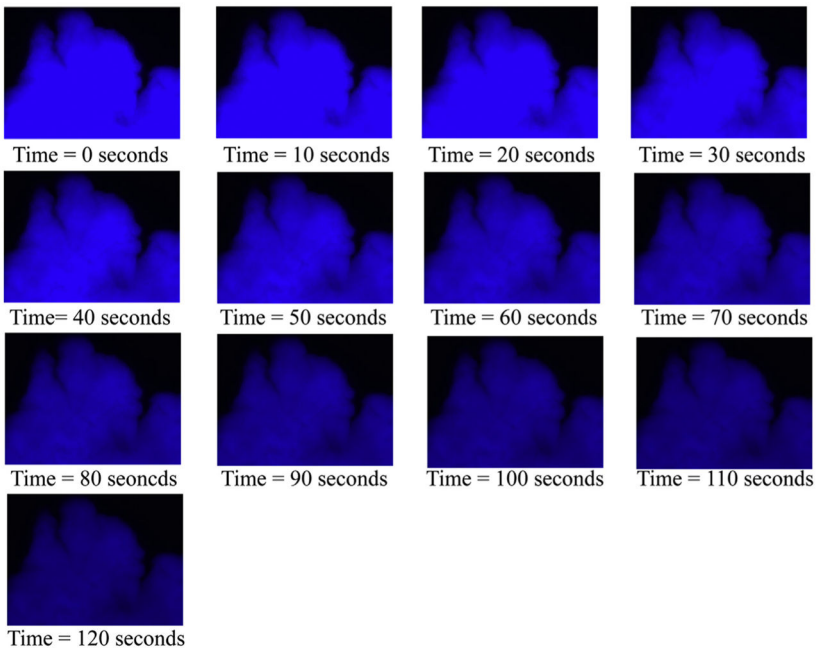

Fig. 2.

Time lapse series of fluorescent images (taken every 10 s for 2 min, starting at time 0) of a sample of mycobacteria killed with BD Perm/Fix. The corresponding brightfield image can be seen in Fig. 1.

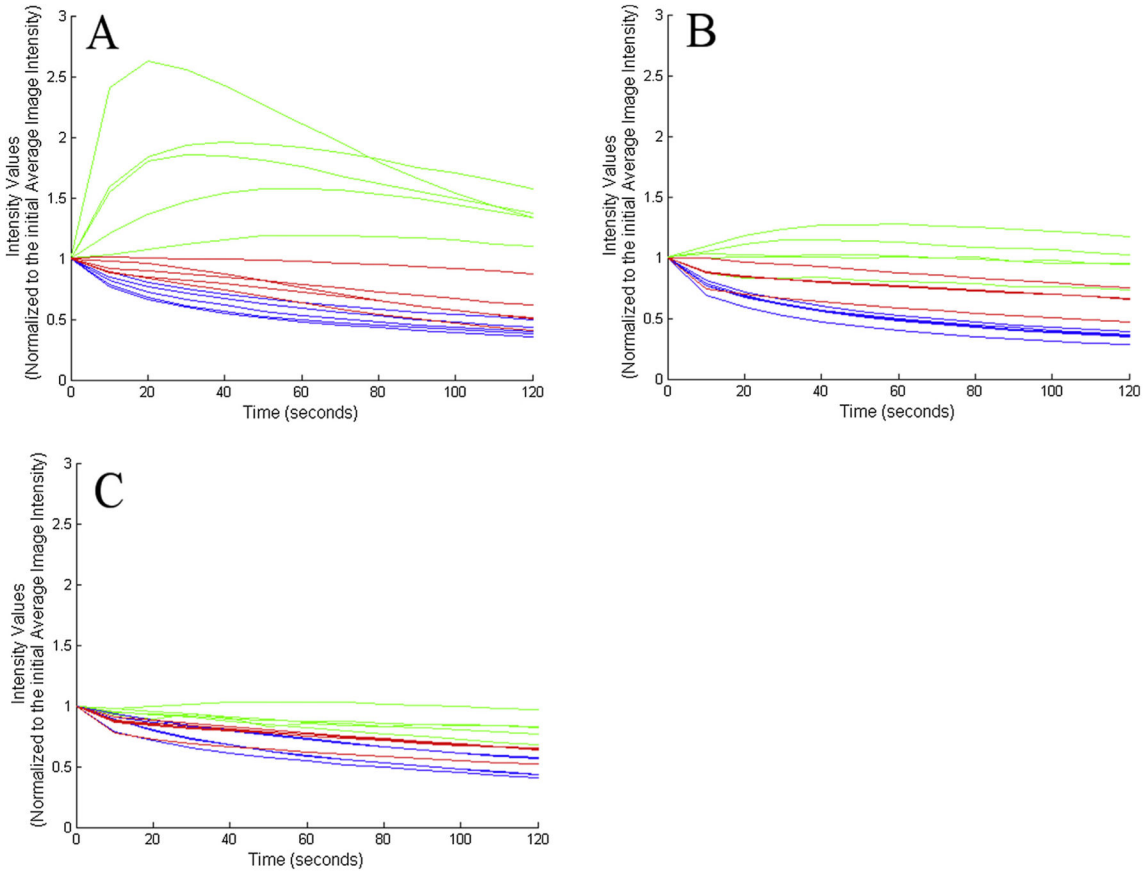

Fig. 3.

Normalized average intensity values for three experiments. Top left is the data from experiment 1 (A), top right is the data from experiment 2 (B), and bottom left is the data from experiment 3 (C). Green lines represent the live mycobacteria, blue lines represent the mycobacteria killed with 70% ethanol, and red lines represent mycobacteria killed with BD Perm/Fix. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

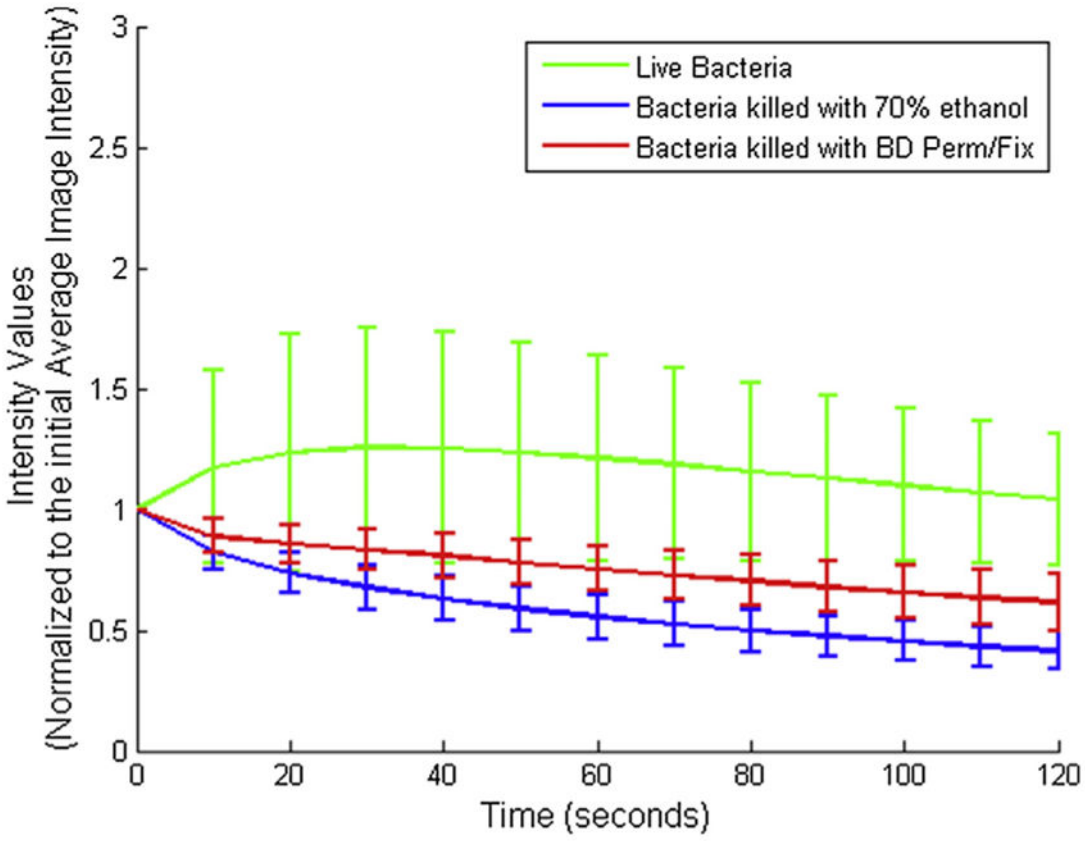

Fig. 4.

Average normalized intensity values over the three experiments with standard error bars. For each variation of mycobacteria, the values at each time point represent the mean value of all 45 samples over the three experiments. Error bars represent the standard deviation around each time point.