Classification of Microbial Samples Using Two-Color Line-of-Sight Fluorescence Imaging

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Abstract: Rapid, low-cost diagnosis of infections is challenging and requires innovation. Two-color fluorescence imaging is proposed to distinguish between microbial species. Microbial smear images show the technique is promising for classifying species *in vitro*.

1. Introduction

Fluorescence spectroscopy is known to have utility in microbial identification and classification [1]. Fluorescence spectroscopy and related techniques including imaging extensions are typically rapid, low-cost, and straightforward to implement, making them a promising tool for microbial identification. However, there have been only a few attempts (for example [2, 3]) to utilize microbial fluorescence for this purpose. Further, widespread lack of quantitative bacterial fluorescence data and analysis introduces a large and unnecessary uncertainty in bacterial fluorescence measurements [4], meaning that much of the existing data in the literature may not be usable for diagnostic design. Here, we propose a fluorescence imaging strategy that targets pyridine nucleotides (e.g., NADH) and flavins (e.g., FAD) based on preliminary spectroscopic analysis. The system is designed to simultaneously excite both NADH and FAD using an ultraviolet light-emitting diode (LED), and the resulting fluorescence is split across two sensors according to wavelength. The LED is directed along the imaging axis, allowing the method to be used on samples where optical access is limited. The remainder of this paper reports on the methods and preliminary results obtained from imaging of a small set of microbial samples.

2. Methods

A two-color near-UV excited fluroescence imaging system was constructed consisting of two monochrome complementary metal-oxide semiconductor (CMOS) sensors (ThorLabs, CS165MU1) and a dichroic beamsplitter with a 458-nm cutoff wavelength (Semrock, FF458-Di02). The 458-nm cutoff wavelength was determined to be appropriate to distinguish between species based on previous spectroscopy measurements. The imaging system is combined with a 2W, 365-nm LED source (ThorLabs, M365LP1) using a 389-nm dichroic beamsplitter (Semrock, FF389-Di01). The LED is focused onto the object plane with a peak irradiance of approximately 30 mW/cm^2. The imaging system uses a 50-mm focal length $f/2$ achromatic lens (Edmund Optics, #65-976) shared by the sensors.

Microbe cultures were grown on nutrient agar plates at room temperature for 2 weeks before imaging, and were prepared from cultures purchased from Flinn Scientific (*B. cereus*) and Carolina Biological Supply (*B. subtilis*, *E. coli*, *M. luteus*, *P. fluorescens*, *S. marcescens*). The mold sample was collected from bread for comparison. For each experiment, a $\sim$ 2µL sample of bacteria or fungi was scraped from the source and mixed into $\sim$20 µL of distilled water over a $\sim$ 1 cm diameter circle on an anodized aluminum substrate. The samples were allowed to dry completely for $\sim$1 hour, then installed at the object plane of the imaging system.

The cameras and LED were gated simultaneously for 200-500 ms per exposure for 10 exposures per sample. Images of the substrate showed negligible luminescence or reflection compared to the fluorescence measurements so no further corrections were applied. The processed fluorescence images were registered using Matlab's built-in multimodal image registration tools and resampled using bicubic interpolation. The fluorescence intensity ratio (FIR) was calculated as the ratio of the red band (450-700 nm) to blue band (400-450 nm) intensities, and histograms were generated from the illuminated portion of each smear identified using Otsu's method.

3. Results

Between 4 and 7 smears prepared from cultures of each species were imaged using the fluorescence imaging system over a 2 week period. A representative set of sample FIR and red-band fluorescence intensity images is shown in Figure 1, FIR histograms for each sample are shown Figure 2, and summary FIR data is shown in Table 1. The FIR is uniform within each smear (see Figure 1) especially near the center where intensity is brightest.

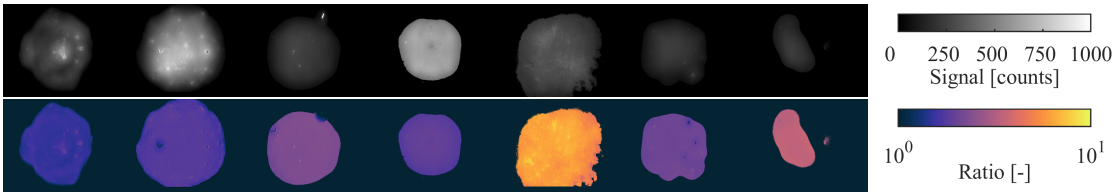

Fig. 1: Red-band fluorescence intensity images (top row) and FIR (bottom row) for one representative sample from each species tested.

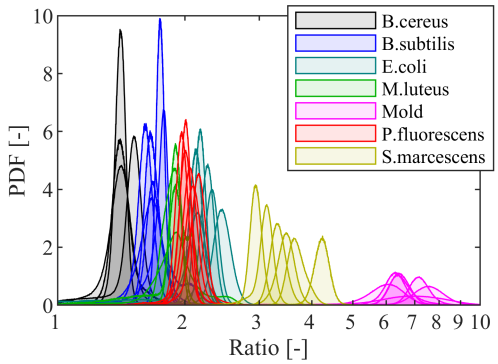

Fig. 2: Measured FIR histogram for 4-7 samples of each species.

Table 1: Summary of measured FIR values for each species.

Species	*n*	**Range [-]**			**Mean [-]**
B. cereus	4	1.40	-	1.51	1.44
B. subtilis	6	1.62	-	1.78	1.69
E. coli	6	2.12	-	2.46	2.25
M. luteus	4	1.89	-	1.91	1.90
Mold	7	6.1	-	7.6	6.69
P. fluorescens	6	1.97	-	2.16	2.05
S. marcescens	6	2.94	-	4.24	3.45

There is some variation in the images that may be due to imperfect image registration. The FIR data may also have some dependence on smear thickness due to radiative trapping but it is not yet clear the extent to which this occurs. The PDF data shows that the majority of FIR images are nearly normal or log-normal distributions; some deviations occur due to artifacts in the images that are not perfectly masked (e.g., dust) and in some cases, multimodal behavior is evident. *M. luteus* smears, e.g., typically have a FIR near 1.9 but have a larger peak as well near 2.1 in some samples. This may be a result of inhomogeneity in the original culture.

Typical PDF widths (full-width at half-maximum; FWHM) are on the order of 10% of the mean FIR value (single-shot FIR precision is ∼5%) with the current object plane pixel size of 15 µm. FIR variability across samples of the same species is somewhat more significant but generally less than 10% except for *S. marcescens* which varies by closer to 20%. *S. marcescens* may exhibit increased FIR sensitivity to cell metabolism and environment compared to other organisms due to the presence of the pigment prodigiosin, the production of which is known to depend on growth phase and available nutrients [5]. Measured FIR values across the limited set of organisms investigated here range from 1.4 (*B. cereus*) to 7 (green bread mold). Although there is overlap in the measured FIR values, the FIR appears to be consistent for a species and significant variation is evident between different species suggesting that the technique may have utility in classifying microbe samples.

4. Conclusion

A two-color, line-of-sight fluorescence imaging technique is proposed for the identification and classification of microbial samples that primarily targets NADH and FAD. Preliminary results show that the FIR varies by a factor of ∼5 across the species tested here, and that there is variation on the order of 10% that may be indicative of metabolic changes in the microbial cultures. Results show the method is promising to distinguish microbial samples, but further effort is needed to characterize additional species and samples.

References

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