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Abstract. Fungi in the *Candida* genus are the most common fungal pathogens. They not only cause high morbidity and mortality but can also cost billions of dollars in healthcare. To alleviate this burden, early and accurate identification of *Candida* species is necessary. However, standard identification procedures can take days and have a large false negative error. The method described in this study takes advantage of hyperspectral confocal fluorescence microscopy, which enables the capability to quickly and accurately identify and characterize the unique autofluorescence spectra from different *Candida* species with up to 84% accuracy when grown in conditions that closely mimic physiological conditions. © The Authors. Published by SPIE under a Creative Commons Attribution 3.0 Unported License. Distribution or reproduction of this work in whole or in part requires full attribution of the original publication, including its DOI. [DOI: [10.1117/1.JBO.22.1.016002](https://doi.org/10.1117/1.JBO.22.1.016002)]

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1 Introduction

Candida species are commensal pathogens that usually reside on mucosal surfaces. If given the opportunity, the fungus will transition from a commensal organism to become a pathogen, which can create infections that range from superficial to systemic. *Candida* species are the most common fungal pathogens, with *C. albicans* arising the most often followed by *C. glabrata* and *C. parapsilosis*, respectively.^{1–4} Not only is there a high mortality rate associated with candidiasis but there is also a healthcare cost that can exceed billions of dollars annually.^{1,5–7}

To reduce mortality associated with fungal infections, early and accurate identification is essential. Studies have shown that late and incorrect diagnosis of *Candida* species leads to a significant increase in mortality.^{8,9} There are multiple clinical diagnostic methods used to support candidiasis diagnoses. Regardless of the detection method (e.g., mass spectrometry and polymerase chain reaction), a microbiological culture step is usually required prior to pathogen identification. This culture step can take between 2 and 5 days from receipt of a clinical sample (e.g., blood, catheter tip, sputum, and urine) for microbiological identification. This long time-to-identification can lead to delays in initiation of optimal antimicrobial chemotherapy. Previous studies have shown relatively poor sensitivity of clinical diagnostics for candidiasis, with a 30% to 50% false negative rate for blood cultures in patients with autopsy-confirmed cases of candidiasis.^{10,11} This demonstrates the limitations of both time and reliability of diagnosis for common existing diagnostic approaches for candidiasis.

Cellular autofluorescence has demonstrated potential as a clinical diagnostic method because it is noninvasive and label-free and has the ability to supply morphological and

biochemical information. Studies have shown that autofluorescence emission can be used to detect microbial pathogens, such as *Mycobacterium tuberculosis*¹² and some pathogenic fungi,¹³ whereas other studies have exploited autofluorescence emission to detect cancer cells.^{14,15} Utilizing autofluorescence is possible due to the differences in both structure and biochemistry of the pathogen and/or the biochemical changes in cells and tissue resulting from disease. Recently, fungal pathogens have been shown to emit autofluorescence in the visible spectrum using blue/green excitation wavelengths.¹⁶ We hypothesize that the autofluorescence emission can be used as a means of rapid identification and can be used parallel to traditional methods to provide a guide for appropriate care at an earlier time.

In this study, we use hyperspectral confocal fluorescence microscopy (HCFM) and multivariate spectral analysis methods to resolve multiple autofluorescence spectra in three *Candida* species pathogens. Utilizing these methods, we were able to identify two independent autofluorescence emission spectra in *Candida* species grown under physiologically relevant conditions. The relative abundance of these spectral factors, together with intracellular spatial distribution features, was sufficient to yield species level differentiation in laboratory experiments.

2 Results

We grew lab strain yeasts of *C. albicans*, *C. glabrata*, and *C. parapsilosis* described in Materials and Methods for the purpose of ascertaining if the individual *Candida* species have unique autofluorescence characteristics. In these studies, we initially used three different growth media: yeast extract peptone dextrose (YPD) (a rich fungal medium), Roswell Park Memorial Institute medium (RPMI 1640) with 3-(N-morpholino)propane-sulfonic acid (MOPS), and pooled human serum. The human serum growth condition was chosen for continued experimentation because of its similarity to the growth condition of fungal pathogens in peripheral blood.

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Fig. 1 MCR identified emission spectra of *Candida* species. (a–c) Two emission spectra that were derived from MCR analysis after 16 h of incubation with *Candida* species. (d) Emission spectra of human serum at 0 h incubation and 18 h of incubation to demonstrate that emission factors found in *Candida* samples are not due to emission of human serum. (a–c) Fungal emission spectra are composed from ≥ 80 cells and (d) serum emission spectra composed from 1 sample at 0 h of incubation and 18 h of incubation.

We detected autofluorescence emission from all of the *Candida* species in all growth media. However, there were no significant differences between species grown in YPD and RPMI when looking at the number of spectral factors detected, the shape of the spectral factor, their wavelength of maximum emission, or their relative abundances throughout the fungal cells. In human serum, however, samples exhibited differences in autofluorescence depending on the species. Because this condition most closely matches the nature of clinical samples, we focused our further investigations on autofluorescence components for *Candida* in human serum. The multivariate curve resolution (MCR) analysis resulted in two different emission factors for all species [Figs. 1(a)–1(c)]. Factor 1's spectral shape and peak (550 nm) are very similar across all species, leading to the idea that the three species investigated share a set of common autofluorescent molecules that emit around 550 nm. In contrast, factor 2's spectral shape and peak (600 to 650 nm) vary depending on which species are being observed [Figs. 1(a)–1(c)]. For example, *C. albicans* factor 2 peak is found at 600 nm, *C. parapsilosis* factor 2 peak is found at 650 nm, and, interestingly, *C. glabrata* factor 2 peak is broad (the peak encompasses 600 to 650 nm). When we examined autofluorescent emission from human serum alone, it did not match either factor found in the *Candida* samples and is not an obvious linear combination of the two factors [Fig. 1(d)].

Along with the spectral factors, the MCR analysis also returns concentration maps that indicate the relative abundance

of each spectral factor. To determine if the spatial localizations of autofluorescence emissions were similar, these concentration maps were overlaid to create composite images, where the green and red color channels correspond to the independent concentration map corresponding to spectral factor 1 and 2, respectively. The composite images were then examined to see if the individual factor's emission came from similar spatial localizations. We found that *C. albicans* factors were mostly spatially separated, whereas both *C. glabrata* and *C. parapsilosis* factors were found to be localized in the same general area within the cells. Next, we compared the spatial localization patterns between species and found unique patterns for each species (Fig. 2). In the case of *C. albicans*, we found very small features mostly composed of factor 1 that were on average around $0.42 \mu\text{m}^2$. For *C. parapsilosis*, we observed large features primarily composed of factor 2 that are on average around $1 \mu\text{m}^2$. However, features in *C. glabrata* images contained both factor 1 and factor 2 with an average area around 0.27 and $0.67 \mu\text{m}^2$, respectively. Remarkably, the majority of autofluorescence from *C. parapsilosis* comes from factor 2. Mean factor 2 intensity of the *C. parapsilosis* features was twofold greater than *C. glabrata* features and tenfold greater than *C. albicans* features. Similar trends are also observed when quantifying the other characteristics of *C. parapsilosis* autofluorescence. These differences in autofluorescence emission geometry as well as other autofluorescence characteristics allowed us to create a classifier from the spatial

Fig. 2 Representative R-G composite images of two autofluorescence factors of *Candida* species. The green channel reflects factor 1 signal and the red channel reflects factor 2 signal. Red boxes are to highlight autofluorescent feature within the fungal cells. Color channels in each image are scaled independently of each other, using a minimum to maximum scale to enhance visualization. Image size and magnification are the same for all images.

and spectral features of the autofluorescence emission that are unique to individual species.

To create an image diagnostic algorithm based on the autofluorescence emission, we quantified multiple spatial and spectral characteristics of each of the autofluorescence signatures from the feature within the hyperspectral fluorescence images. To validate the accuracy of the classification tree, we measured three statistics for six levels of pruning using the training set (described in Materials and Methods) and plotted the accuracy of both the training set and test set [Figs. 3(a) and 3(b)]. The statistics and accuracy plot revealed some of the less pruned classification trees (i.e., larger classification trees) have a lower true predictive error, demonstrating that the full classification tree may have been overfitting and that the predictions reflected

noise or outliers. The accuracy plot also depicted the pruning level (i.e., size of the classification tree) where the training set and test set diverged. Based on these results, we selected level 4 as the optimal pruning level. From the pruned tree, we were able to achieve 84% accurate species identification from the test set of data [Fig. 3(b)]. We were also able to observe which autofluorescent characteristics were used to construct the classification tree (Fig. 4). Interestingly, in this predictive model, factor 1 autofluorescent characteristics are more important than factor 2 characteristics for predicting the *Candida* species. We then calculated the predictive accuracy for identifying each species in the independent test set. The classification tree was able to predict 75% of *C. albicans* samples accurately, 88% of *C. glabrata* accurately, and 83% of *C. parapsilosis* accurately. On closer inspection of the results, it was found that *C. albicans* was misidentified twice, once with *C. glabrata* and once with *C. parapsilosis*. Whereas *C. glabrata* was found to only be misidentified with *C. parapsilosis* and *C. parapsilosis* misidentified with *C. glabrata*.

3 Discussion

Candida species are well known for their ability to cause morbidity and mortality. It is also known that some species, such as *C. glabrata*, have intrinsic resistances to azole-based antifungal drugs.^{17,18} Due to this, it is important to be able to quickly and accurately identify the pathogenic fungal species prior to administering treatment. The method we describe in this study takes advantage of the spatial and spectral resolution of HCFM. The ability to characterize the unique autofluorescence spectra in the different *Candida* species coupled with their spatial localizations has allowed for species level identification of *Candida* species yeasts grown in conditions that closely mimic pathological conditions and commonly available clinical specimen types.

The serum growth condition was chosen and designed to be a model of growth conditions in blood. Most of the samples assayed for *Candida* in diagnostic reference laboratories are peripheral blood (~96% of specimens in our clinical isolate library) and the remainder are mostly medical devices in intimate contact with central circulation. Serum is a reasonable and tractable model for blood in that it contains all noncellular blood components except clotting factors. It is commonly used to study the response of *Candida* to growth in conditions that mimic growth in systemic circulation. Indeed, *Candida* species are known to respond to growth in serum with

Fig. 3 Classification tree pruning. (a) Overfitting decreases the accuracy of the classification tree for the test set. Pruning level for classification tree was chosen by the divergence of the test set and training set, which corresponds with pruning level 4 (i.e., a 12 node classification tree). (b) Table displaying statistics for the different pruning levels of the classification tree. Bold statistics indicate chosen level for prediction analysis of the test set.

Fig. 4 Feature characteristics are used to accurately predict species. The pruned classification tree indicates that factor 1 characteristics are crucial for accurate species identification.

significant physiological changes, such as alterations of cell wall components¹⁹ as well as germination of filamentous cells in *C. albicans*. Therefore, we designed this condition to test whether growth in conditions mimicking those of clinical specimens would induce physiological changes in *Candida* that might alter its autofluorescence. These conditions would be more representative of the autofluorescence spectrum of *Candida* to be detected in clinical specimens relative to standard fungal culture medium growth conditions. Continuation of these studies into whole blood and direct visualization on catheter tips would be productive and promising ways to extend this work in the future.

Emission of autofluorescence in fungi is commonly due to native fluorophores, such as flavins.²⁰ Flavins and flavoproteins likely contribute to the fungal autofluorescence that we observed. Flavin compounds emit around 530 nm from 460 nm excitation,²¹ which match closely with our factor 1 peak wavelength and our excitation line. Flavin molecules also associate with the plasma membrane, intracellular granules, and mitochondria.²² We did not observe any autofluorescent signatures around the plasma membrane, but we did observe intracellular signatures for factor 1. For both *C. albicans* and *C. glabrata*, we detected small features for factor 1 leading to the possibility that we observed flavin molecules either in intracellular granules or mitochondria. Interestingly, when observed, factor 1 tended to be smaller in size than factor 2. Also, factor 1 tended to be located inside areas that also contained factor 2, leading us to speculate that factor 2 may be another native fluorophore, yet to be described, that occupies a similar subcellular compartment.

By utilizing autofluorescent emission characteristics, the classification tree is able to accurately predict the *Candida* species, demonstrating the robustness of the procedure as a quick and accurate identification method. Under current conditions, the classification tree can identify 84% of samples accurately. It is generally appreciated that cell size varies in the *Candida* genus. For example, *C. parapsilosis* is on average 2 to 4 times larger than *C. glabrata*.²³ However, in this current

experiment, it was not possible to measure the area of the fungal cells with accuracy in an automated fashion. Future experiments with a membrane-specific contrast agent added to the medium just prior to imaging could improve the accuracy by incorporating information on the size of the cells.

Previous studies have shown that by utilizing a single excitation source coupled with a monochromator and correlating microbial sample type with the intensity of emission at several wavelengths, it was possible to discriminate bacterial and fungal species.¹⁶ In contrast, our approach uses the entire emission spectrum from 500 to 850 nm to identify multiple component spectra in the specimens. Our results also extend earlier approaches by measuring autofluorescence events at the individual cell level, whereas other methods use a population-based approach and measure the overall autofluorescence signature of a population of cells. Finally, our approach utilizes identifying information from the subcellular spatial distribution of autofluorescence signals. Other molecular methods, such as fluorescent *in situ* hybridization (FISH), have been described both as quick and accurate.²⁴ However, utilizing FISH requires that each species needing identification has its own unique probe as well as preparing the sample in such a way that the probe can enter the cell and a culture step to generate enough sample to work with. Because our method does not require large numbers of cells and is a label-free method, it may be usable on a minimally processed clinical specimen, perhaps even as a point of care diagnostic.

As a potential method for identifying fungal pathogens, we provide comparisons to published data on performance of other diagnostic methods currently in use. These comparisons illustrate the advantages, primarily in terms of speed of diagnosis, of our method over certain current limitations with commonly used methods. However, we also point out that detection of fungi by autofluorescence is best thought of as an adjunctive diagnostic test to classical microbiology. These methods will still be required to confirm microbiological identification and to measure drug sensitivities. We think that as the autofluorescence method matures, more detailed effectiveness comparisons

with other technologies would be a worthy subject of future investigation.

In summary, currently deployed fungal species identification methods based on culture can range from 2 to 5 days depending on the method being used, which can cause complications related to delays in treatment. The laboratory investigation conducted demonstrates a 75% prediction accuracy for *C. albicans*, 88% prediction accuracy for *C. glabrata*, and 83% prediction accuracy for *C. parapsilosis* in clinically relevant media. The next step to determine robustness of this method will be to include clinical samples. Our work represents the first step toward developing a label-free method for rapid, culture-free identification of fungal species. If used parallel with traditional methods it could provide a guide for appropriate care at an earlier time and could reduce morbidity and mortality.

4 Methods

4.1 Yeast Growth/Preparation

C. albicans (ATCC, MYA-2876), *C. glabrata* (ATCC, 2001), and *C. parapsilosis* (ATCC, 22019) were grown from glycerol stock, stored at -80°C . Samples were grown in YPD, RPMI-1640 + 0.165M MOPS, or human serum (EMD Millipore, S1) for 16 h at 37°C in an orbital shaker at 250 rpm. Yeast were then put on microscope slides and sealed with nail polish.

4.2 Hyperspectral Confocal Fluorescence Microscopy

A custom built HCFM was used to acquire the autofluorescent emissions from the samples. Previous work describes the methodology for image acquisition using the custom built microscope.²⁵ In summary, a 488-nm laser (Coherent, Inc. Sapphire) and a 60 $\times$ apochromat objective (Olympus Plan Apochromat, NA 1.4) were used to excite fluorescence from a diffraction-limited spot. The laser power was $\sim 80\ \mu\text{W}$ at the entrance to the microscope and the integration time was 0.2 ms/pixel. To detect fluorescent emissions, a prism spectrometer coupled to an electron multiplying charged-coupled device camera (Andor Technologies, Inc., iXon) was used.

4.3 Spectral Analysis

All images were preprocessed to remove known spectral artifacts introduced by the imaging system (cosmic ray spikes, detector offset, and structured dark noise),²⁶ as well as to calibrate the wavelength axis. The resulting individual spectral images were combined into three composite image data sets, one for each *Candida* species. MCR was performed on each of these composite data sets to develop a spectral model that described more than 98% of the spectral variance. The MCR algorithm has been described previously^{27,28} and has demonstrated success in exploratory analysis to identify underlying spectral components from multicomponent biological systems.^{29,30} The MCR results identified near-identical spectral components regardless of the yeast species; therefore, a combined spectral model for all three species was deemed appropriate. This combined model was generated by performing MCR on a combined set of images from all three species and described >96% of the spectral variance in the data. Classical least squares prediction was used to determine the location and abundance of each spectral component in the spectral images. Images corresponding to the location and abundance of individual spectral

components were exported as Tif files for subsequent image processing.

4.4 Classification

Fiji (ImageJ) was used to threshold and quantify features. A threshold of three standard deviations above background intensity was used to extract features for all emission factors. Quantification of features was measured for total and average intensity per feature, average intensity variance per feature, total and average number of features per cell, and total and average area of features describing the nature of the autofluorescence in the cells. The characteristics were then compiled and input into a binary classification tree algorithm. A classification tree analysis was developed in MATLAB[®] using the statistics, and a machine learning toolbox (Mathworks, version 2015a) was then used to create the classification tree and accuracy statistics. To test the performance of the classifier, we divided the data into a training group of 337 cells and a test group of 37 cells (composed of 10% of each species population). To validate the accuracy of the classification tree, we measured three statistics: cross validation error, resubstitution error, and the true predictive error for six levels of tree pruning using the training set. To calculate the true error of prediction, the number of misclassifications in the test set was divided by the total number of samples in the training set. All data presented were pooled from triplicate biological replicates of samples.

Disclosures

The authors disclose that there are no financial interests or conflicts of interest.

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