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Optical Spectroscopy on Fungal Diagnosis

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1. Introduction

Occurring globally, most fungi are undetectable to naked eye, living for the most part in soil, dead matter, as well as symbionts of plants, animals, or other fungi. Fungal infections are of important concern in several patients submitted to treatment with prolonged antibiotic therapy, immunosuppressive drugs, corticosteroids, degenerative diseases, diabetes, neoplasias, blood dyscrasias, endocrinopathies and other debilitating conditions as transplanted patients. In the most of cases, a rapid diagnostic and treatment is therefore critical (Davies, 1988).

In dermatological mycology, diagnostics modalities available are histopathology, direct microscopic examination of clinical specimen, culture and serology. Visual examination of skin, nail and hair samples for detect the presence of fungi is an essential step to confirm the clinical diagnosis of cutaneous fungus infection. Normally, the identification of fungi is done mainly by morphological *in vivo* studies based on visual macroscopic and microscopic aspects. Therefore, visual inspection requires a lot of training.

Characteristics of an organism's growth on culture media, such as colony size, color, and shape, provide clues to species identification. The prolonged incubation time is a major limitation of fungal cultures as a diagnostic tool. Biochemical and molecular biology techniques such as serology are also used for that purpose (Rippon, 1999; De Hoog et al., 2001). In particular, the serology test for detection of fungal antibodies take about 2 to 3 weeks, and it is of limited value especially in immunocompromised patients in whom production of antibodies is impaired. Multiple test techniques can be highly accurate but may require several days to yield results, creating delay in diagnosis, which may even culminate in a fatal outcome.

Here we exploit the autofluorescence spectroscopy of fungi as a tool to identify microbial infections. Different types of light source were used to excite endogenous fungal fluorochromes and a simple mathematical method was developed to identify specific features of the emission spectrum of six fungal species.

2. Fungal autofluorescence

Fluorescence consists of the electromagnetic radiation emitted by a material, especially of visible light, after absorption of incident radiation and persisting only as long as the stimulating radiation is continued. A number of cellular constituents fluoresce when excited directly or excited by energy transfer from another constituent, this fluorescence is called autofluorescence (Prasad, 2003). In the most of cases, excitation can be obtained by use of near ultraviolet (UV) light, with wavelength (λ) going from 320 to 400 nm (Prasad, 2003; Richards-Kortum & Sevick-Muraca, 1996). After the absorption of UV light by a fluorochrome, radiation of longer wavelength (visible light) is emitted.

The autofluorescence spectroscopic technique is a simple and quick procedure that can be exploited on fungal detection from *in vivo* diagnosis of dermatophytic infection to *in vitro* tissue or incubation on culture media by immunofluorescent techniques (Mustakallio & Korhonen, 1966; Asawanonda & Charles, 1999).

The first use of fluorescence by UV excitation in dermatology was reported in 1925 (Margarot & Deveze, 1925), with the detection of fungal infection on hair. At the present time UV light in dermatology is used predominantly in diagnostic areas involving pigmentary disorders, cutaneous infections, and the porphyrias (Asawanonda & Charles, 1999). Moreover, UV light can be very helpful establishing the extent of infection by *Malassezia furfur*, which presents a yellowish autofluorescence (Mustakallio & Korhonen, 1966). Blue-green fluorescence can be observed in *Microsporum audouinii* and *Microsporum canis* infections (Asawanonda & Charles, 1999). *Microsporum distortum* and *Microsporum ferrugineum* also present a greenish fluorescence. A faint blue color is emitted by *Trichophyton schoenleinii* and a dull yellow is seen in *Microsporum gypseum* fluorescence (Asawanonda & Charles, 1999). *In vitro* studies indicate that the chromophores pteridine is one of the chemical substances responsible for the fluorescence of *M. canis* and *M. gypseum* (Wolf, 1957; Chattaway & Barlow, 1958; Wolf, 1958). It was also showed the tryptophan dependence on the fluorochrome synthesis of *Malassezia* yeasts (Mayser et al., 2004; Mayser et al., 2002).

The advantages and limitations of UV light on fungal diagnosis are already known (Asawanonda & Charles, 1999). The emission spectrum overlap of different fungi can make them indistinguishable by a visual inspection of fluorescence. Moreover, some species of fungi do not contain fluorescent chemicals and therefore not all the fungi infections can be detected by visual analyses of their autofluorescence. To overcome this limitation nonspecific fluorochrome stains, such as Calcofluor White ($\lambda \sim 440\text{nm}$) and Blancophor ($\lambda \sim 470\text{nm}$) that binds to cellulose and chitin in cell walls of fungi, can be used to detect without ambiguity fungal elements in dermatological assays (*in vitro*) (Harrington & Hageage 1991).

3. UV Light Sources

In dermatology, the long-wave ultraviolet (UV) light source, known as Wood's lamp, has become an invaluable tool for diagnostic procedure. Wood's lamp was invented in 1903 by Robert W. Wood (1868–1955) (Wood, 1919). Wood's lamp is a high-pressure mercury fluorescent lamp that emits a broad band spectrum, with wavelength going from 320 to 400 nm, with a peak at 365 nm. In fluorescent lamps, mercury atoms are excited through

collisions with electrons and ions. When the atoms return to their original energy level, they emit photons. The output intensity of a Wood's lamp is typically of few mW/cm².

For medical purposes, light on the UV region of the electromagnetic spectrum can be obtained with optoelectronics devices rather than Wood's lamp. A light-emitting diode (LED) is a semiconductor device that generates light when an electric current passes through it. LEDs are completely solid-state technology, making them extremely durable. On other hand, vibration or shock easily breaks the fragile glass tubing of a fluorescent lamp. In addition to being robust and efficient producers of light, LEDs are compact, low voltage and low power consuming devices, suitable to be used in small equipments. Moreover, it is possible to find LEDs in a wide range of colors, extending from ultraviolet (350 nm) to the far-infrared (1500 nm) region of the electromagnetic spectrum.

Ultraviolet light can also be obtained by the use of medical LASER systems, as excimer LASER (XeCl, XeF) and by infrared pulse LASERS (exploring the generation of second and third harmonic). The number of LASERS in medical clinics has rapidly increased in the last two decades, turning LASER therapy and diagnostic more accessible.

4. Autofluorescence spectroscopy

This section is devoted to describe the possibility of applying different UV light sources on the identification of fungi by optical spectroscopy.

Here six species of filamentous fungi were used: Five dermatophytes (*Microsporum gypseum*, *Microsporum canis*, *Trichophyton schoenleinii*, *Trichophyton rubrum*, *Epidermophyton floccosum*) and one *hyalohyphomycetes* (*Fusarium solani*). These fungi are recognized as emergent pathogens in the Northeast of Brazil. All of the samples studied were isolated from patients with dermatomycoses (superficial mycoses) attended in the medical mycology laboratory at the Federal University of Pernambuco. The biological materials were cultivated on Petri dishes with a Sabouraud Dextrose Agar (SDA) medium containing chloranphenicol (0.05 g/L). After isolation and identification (by microscopic and macroscopic morphological analysis) of the fungi, the samples were placed in glass tubes with SDA without antibiotic and preserved at room temperature (25°C).

Four different light sources were explored in the experiment: 4 Watts UV fluorescent lamp (Wood's lamp) from Toshiba (BLUE FL4BLB) and from XELUX (G5), UV LEDs from Roithner LASER (UVLED365-10), and the third harmonic from a Nd:YAG nanosecond pulsed laser (Continuum/ Surelte). The light sources spectra are presented on figure 1. All light sources radiates on the UV-A region of the electromagnetic spectrum. The bandwidth and the peak wavelength of the light sources used were respectively 43 and 353nm for the Toshiba lamp, 18 and 375nm for the Xelux lamp, 19 and 363nm for the Roithner LED. The bandwidth of the UV LASER light at 352 nm was 3.5nm.

Fig. 1. Light sources emission spectra

In our experimental setup for fungal autofluorescence spectroscopy, the excitation UV light was focused on the sample. To keep the UV intensity with about the same value ($5\text{mW}/\text{cm}^2$) for all light sources, neutral density filters were used. The fungal autofluorescence light was collected by a lens system and sent to a spectrometer (SPEX/Minimate). A color filter (Corning 3-73) was placed at the entrance of the spectrometer to ensure that the excitation light would not reach the photomultiplier. A GaAs photomultiplier (RCA Electronic Device) was used to convert the collected light to an electrical signal. The signal was digitalized by a lock-in amplifier (SR530 Stanford Research) and sent to a computer, where it was stored and analyzed. The spectrum resolution of the experimental system was 0.5 nm . The experimental setup scheme is shown in Figure 2.

Fig. 2. Scheme of the experimental setup used.

For all fungi, the first fluorescence measurements were taken seven days after inoculation, and repeated 14 and 21 days later. In all experiments, all samples were investigated applying different light sources (UV Lamp, LED, and LASER). The excitation light power was monitored to ensure similar excitation conditions. Two set of attempts were performed

one month apart. Spectroscopic analysis of isolated growth medium fluorescence was also performed.

5. Spectroscopic results and analysis

All samples studied fluoresced. Figure 3 shows the fluorescence of *F. solani*, *T. rubrum* and *T. schoenleinii*, *M. gypseum*, *M. canis* and *E. floccosum* excited with the UV LED 21 days after inoculation. An increase of the fluorescence intensity at the 21st day was noticed for several fungi. Spectroscopic results show (Figure 4) that fluorescence emissions induced by UV Lamps (Toshiba and Xelux) are quite similar from the ones obtained with UV LED (Figure 3). It can be observed on Figure 3 and 4 that the *T. rubrum* has a fluorescence spectrum very distinct from the other microorganisms.

Fig. 3. Fungal emission after UV LED excitation (21 days after inoculation).

Fig. 4. Fungal emission after UV lamp excitation (21 days after inoculation).

The closeness of the peak emission wavelength and the spectrum shape of all samples, other than the *T. rubrum*, make it hard to distinguish fungi by a visual analysis of their autofluorescence. Although a careful spectroscopic analysis of the fluorescence shows distinct features on the detected emissions. Difference between fungi fluorescence spectra can be better perceived by analyzing the emission intensity at specific wavelength. Table 1 presents the values of a relative intensity defined as $(I_{\lambda 1}-I_{\lambda 2}-I_{\lambda 3})/(I_{\lambda 1}+I_{\lambda 2}+I_{\lambda 3})$, where $I_{\lambda 1}$, $I_{\lambda 2}$ and $I_{\lambda 3}$ are respectively the intensity of the fluorescence at 430, 485 and 550 nm, obtain in Fig. 3.

-----	$(I_{\lambda 1}-I_{\lambda 2}-I_{\lambda 3})/(I_{\lambda 1}+I_{\lambda 2}+I_{\lambda 3})$	
Fungi	1° experiment	2° experiment
<i>T. rubrum</i>	1.25 ± 0.05	1.25 ± 0.05
<i>F. solani</i>	-4.15 ± 0.05	-4.20 ± 0.05
<i>T. schoenleinii</i>	-3.50 ± 0.05	-3.50 ± 0.05
<i>M. canis</i>	-7.50 ± 0.05	-7.55 ± 0.05
<i>E. floccosum</i>	-1.60 ± 0.05	-1.60 ± 0.05

Table 1. Fungal fluorescence spectral characteristics (LED excitation).

Fungal emission spectra obtained using the narrow band UV LASER excitation source is presented in Figure 5. UV LASER induced fluorescence present three peaks, resulting from specific energy decaying channels. The graphics in Figure 5 are normalized at 460 nm, to better show the spectra distinctions. Relative intensities of the peaks (417, 460 and 505 nm) can be explored to identify the studied fungi. Table 2 presents values of a relative intensity defined as $(I_{\lambda 1}-I_{\lambda 2})/(I_{\lambda 1}+I_{\lambda 2})$, where $I_{\lambda 1}$ and $I_{\lambda 2}$ are respectively the intensity of the fluorescence at 417 and 505nm, obtain by Fig. 5. The results in Table 2 indicate that a spectroscopic analysis of UV LASER induced fluorescence can be explored on the identification of fungi.

Fig. 5. Fungal emission after UV LASER excitation (21 days after inoculation).

-----	$(I_{\lambda 1}-I_{\lambda 2})/(I_{\lambda 1}+I_{\lambda 2})$	
Fungi	1° experiment	2° experiment
T. rubrum	0.55	0.54
F. solani	-0.09	-0.13
T. schoenleinii	-0.13	-0.13
M. canis	-0.10	-0.08
E. floccosum	0.28	0.26

Table 2. Fungal fluorescence spectral characteristics (LASER excitation)

6. Conclusion

Here it was demonstrated that optical spectroscopy can be exploit as a tool for fungal diagnosis. We observed distinct features on fungal emission. Although it is impossible do distinguish several microorganisms only by a visual analysis of their fluorescence. A careful spectroscopic analysis of fungal autofluorescence is required on the on the identification of microbial infections.

By exciting fungal fluorochromes with a broadband UV light source (LED and Lamp), we showed that a multiple wavelength (430, 485 and 550 nm) analysis of their autofluorescence spectrum can differentiate several fungi species. It has been proposed before a device for fungal diagnosis base on the analysis of the relative intensity of two specific wavelengths (Rativa, 2008). Here a better method for fungal identification is presented, based on the evaluation of three spectra regions of the fluorescent emission. We believe that a more refine mathematical model should be explored on a bigger group of fungi species. For that we are currently working with the Kolmogorov-Smirnov method on the fungal autofluorescence spectrum.

Here we also showed that UV narrow band light source (LASER) can lead to special features on the fluorescent spectrum. In that case a dual-wavelength analysis can be use to distinguish all the six fungal species studied. Although UV LASERS are still too expensive to be used for fungal diagnosis, their prices are constantly decreasing over the past decade. In the near future UV LASER can be an important tool for dermatological diagnosis.

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