Revista Brasileira de Engenharia Biomédica,
v. 23, n. 1, p. 25-30, abril 2007

ISSN 1517-3151

Artigo Original
Recebido em 06/10/2006, aceito em 13/03/2007

Perspectives on *in vitro* fungal diagnosis with UV light

Perspectivas no diagnóstico de fungos in vitro com luz UV

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Abstract
The growing incidence of microbial infections and the increasing ability of such organisms to acquire resistance to antimicrobial treatment lead the requirement of fast bacteria and fungi identification methods. In dermatology optical techniques that explore ultraviolet light, as the Wood's method, are important tools for diagnostic procedure. In this work we exploited optical fluorescence spectroscopic techniques on fungal identification. The applied method utilizes different ultraviolet light sources (lamp, LED, LASER) to optically excite the fluorochromes of several fungi (*in vitro*). Six species of fungi (*Microsporum gypseum*, *Microsporum canis*, *Trichophyton schoenleinii*, *Trichophyton rubrum*, *Epidermophyton floccosum* and *Fusarium solani*) were analyzed. We show that a simple and refined spectroscopic analysis of the light emitted by fungi can overcome the Wood's technique limitations on the identification of fungi infections. The results indicate that by analyzing the bandwidth and the peak wavelength of the fungal fluorescence one can distinguish different microorganisms. We also show that different features from each fungal fluorescence do not change with the use of Wood's lamp or ultraviolet LEDs as the excitation sources. Moreover, we demonstrate that ultraviolet LEDs and LASERs can be applied in fungal identification, just as Wood's lamps in clinical diagnoses of fungal infection.
Keywords: Fluorescence, Fungal infection, UV light, Wood's lamp.

Resumo
O aumento na incidência de infecções fúngicas e a crescente habilidade dos organismos em adquirirem resistência a tratamentos antimicrobiais tornam necessária a introdução de novos métodos de identificação rápida de fungos e bactérias. Em dermatologia, técnicas ópticas que exploram luz ultravioleta, como o método de Wood, apresentam-se como importantes ferramentas para o diagnóstico clínico de infecções dermatológicas. Neste trabalho são exploradas técnicas de espectroscopia óptica por fluorescência na identificação de fungos. O método aplicado utiliza diferentes fontes de luz ultravioleta (lâmpada, LED, Laser) para excitar opticamente cromóforos de alguns de fungos (in vitro). Seis espécies de fungos (Microsporum gypseum, Microsporum canis, Trichophyton schoenleinii, Trichophyton rubrum, Epidermophyton floccosum e Fusarium solani) foram investigadas. Os resultados obtidos demonstram que algumas das limitações no diagnóstico de fungos com lâmpada de Wood podem ser superadas utilizando análises espectroscópicas refinadas. A largura espectral e o comprimento de onda de pico da fluorescência emitida pelos fungos são parâmetros que podem ser usados na distinção de microorganismos de espécies diferentes. Foi verificado também que, com a excitação óptica de fungos por LEDs, a luz emitida pelos microorganismos apresenta as mesmas características espectrais da fluorescência obtida com a excitação por uma lâmpada de Wood. Os resultados aqui apresentados indicam a possibilidade do uso de LEDs e LASERs no diagnóstico clínico de infecções fúngicas.
Palavras-chave: *Fluorescência, Infecção fúngica, Luz ultravioleta, Lâmpada de Wood.*

Introduction

More than one million species of fungi are known in nature. 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.

The identification of fungi is done mainly by morphological studies based on visual macroscopic and microscopic aspects. Biochemical and molecular biology techniques are also used for that purpose (de Hoog *et al*., 2001; Rippon, 1988).

Optical fluorescence techniques can be exploited on fungal detection. Fluorescence consist of the light emitted by a material (some molecules of fungi, for instance) after its quantum energy state has been excited. In several molecules, excitation can be obtained by use of near ultraviolet (UV) light, with wavelength ranging from 320 to 400 nm (Prasad, 2003; Richards-Kortum and Sevick-Muraca, 1996). After the absorption of UV light by a fluorochrome, radiation of longer wavelength (visible light) is emitted. The first use of ultraviolet light in dermatology was reported in 1925 (Margarot and Deveze, 1925), with the detection of fungal infection on hair.

UV light can be very helpful establishing the extent of infection by *Malassezia furfur*, which presents a yellowish fluorescence (Mustakallio and Korhonen, 1966). Blue-green fluorescence can be observed in *Microsporum audouinii* and *Microsporum canis* infections (Asawanonda and Taylor, 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 and Taylor, 1999). Overlapping of emission spectrum of different fungi can make them indistinguishable by a visual inspection of fluorescence.

Not all dermatophytes present fluorescence that is easily perceived by visual inspection. Fluorescence is usually seen on dermatophytes member of the *Microsporum* genus. *In vitro* studies indicate that the chromophores pteridine is one of the chemical substances responsible for the fluorescence of *M. canis* and *M. gypseum* (Chattaway and Barlow, 1958; Wolf, 1957; Wolf, 1958). Moreover, recent investigations showed the tryptophan dependence on the fluorochrome synthesis of *Malassezia* yeasts (Mayser *et al*., 1998; Mayser *et al*., 2002; Mayser *et al*., 2004).

UV lamp is an important tool for diagnostic procedure in dermatology. Wood's lamp is a high-pressure mercury fluorescent lamp that emits a broad band spectrum, with wavelength ranging 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/cm^2. Wood's lamp is not used only on the detection of fungal and bacterial infection, but it is also applied on the diagnosis of porphyrias and pigmentation disorders as vitiligo and melasma (Asawanonda and Taylor, 1999).

For medical purposes, light on the UV region of the electromagnetic spectrum can be obtained with optoeletronics 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, so extremely durable (their lifetime is about 100,000 hours). On other hand, vibration or shock easily breaks the fragile glass tubing of fluorescent lamps. In addition to being robust and efficient light emitters, LEDs are compact, low voltage and low power consuming devices, suitable to be used in small equipment. Moreover, it is possible to find LEDs in a wide range of colors, extending from ultraviolet (350 nm) to the far-infrared (1,500 nm) regions of the electromagnetic spectrum.

Ultraviolet light can also be obtained by means of medical Laser systems, as excimer Laser (XeCl, XeF) and by infrared pulsed Lasers (exploring the generation of third harmonic) (Siegman, 1986). The number of Lasers in medical clinics has rapidly increased in the last two decades, turning Laser therapy and diagnostic more accessible.

The aim of this work is to study the possibility of applying different UV light sources on the identification of fungi. We show that light emitted by fungi upon LED excitation is similar to the fluorescence induced by Wood's lamp. The advantages and limitations of Wood's lamp on fungal diagnosis are already known (Asawanonda and Taylor, 1999). Moreover, we show that simple refined spectroscopic analysis of fluorescence can overcome the Wood's technique limitations on the identification of fungi infections.

Materials and Method

Organism and culture conditions

For all experiments six species of filamentous fungi were used: five dermatophytes (*Microsporum gypseum*,

Microsporum canis, *Trichophyton schoenleinii*, *Trichophyton rubrum* and *Epidermophyton floccosum*) and one hyalohyphomycetes (*Fusarium solani*), nowadays recognized as an emergent pathogen.

All studied samples 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 of the fungi (by microscopic and macroscopic morphological analysis), the samples were placed in glass tubes with SDA without antibiotic and preserved at room temperature (25 °C).

Experimental setup

Scheme of the experimental setup used is shown in Figure 1. Three different light sources were explored in the experiment: a 4 W UV fluorescent lamp (Wood's lamp) (Toshiba blue FL4BLB and Xelux G5), UV LEDs (Roithner Laser UVLED365-10), and the third harmonic from a Nd:Yag nanosecond pulsed Laser (Continuum Surelte). The UV light intensity at the sample was approximately 5 mW/cm^2. To keep the UV intensity within about the same value for all light sources, neutral density filters were used in the Laser system, and five UV LEDs were arranged together.

The bandwidth and the peak wavelength of the light sources used were respectively 43 nm and 353 nm (Toshiba), 18 nm and 375 nm (Xelux), 19 nm and 363 nm (Roithner). The bandwidth of the UV Laser light (352 nm) was 3.5 nm. The excitation light was focused on the sample by a 10 cm convergent lens. The emitted 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 into electrical signal. This signal was digitalized by a lock-in amplifier (Stanford Research SR530) and sent to a computer, where it was stored and analyzed. The spectrum resolution of the experimental system was 0.5 nm. A digital oscilloscope (HP 54501A) and a chopper, placed between the light source and the sample, were used to study the temporal evolution of the fungal fluorescence. For this analysis the LEDs' power was modulated with a duty cycle of 1/10, keeping the light on during 1 μs.

Fluorescence experiment

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

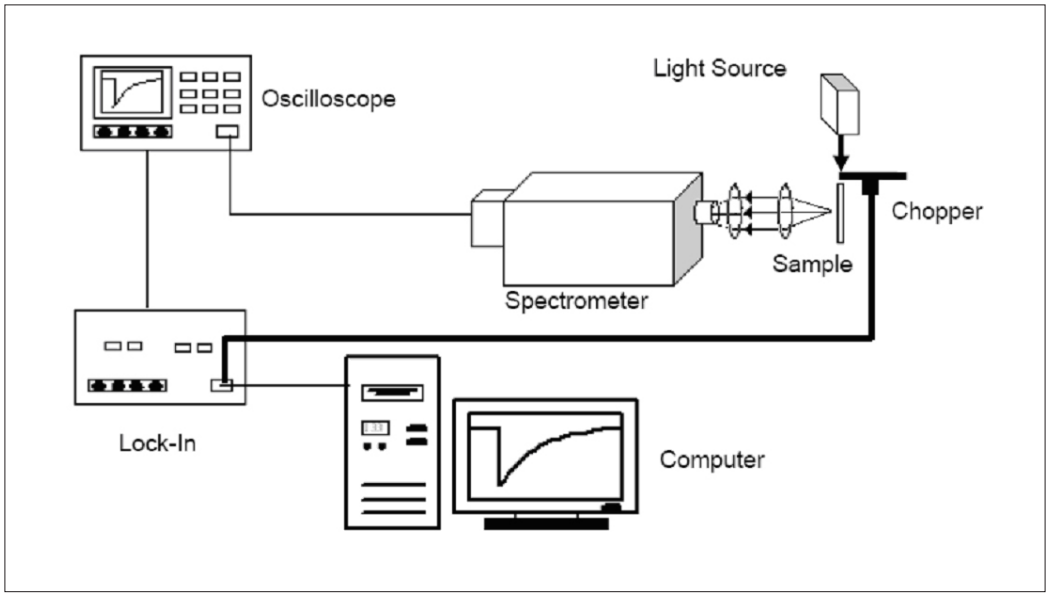

Figure 1. Scheme of the experimental setup used.

Results

All studied samples have fluoresced. Figure 2 shows the fluorescence of the samples excited with the Wood's lamp 21 days after inoculation. For a better visualization, fluorescence spectra are presented in two figures: *Fusarium*, *T. rubrum* and *T. schoenleinii* fluorescence spectra are shown in Figure 2a while *M. gypseum*, *M. canis* and *Epidermophyton* emission curves are presented in Figure 2b.

An increase of the fluorescence intensity at the 21st day was noticed for several fungi. The *T. rubrum* fluorescence spectrum is very distinct from the growth medium emission. Different from all other samples, the *T. rubrum* fluorescence characteristics are established in the first seven days after inoculation. Figure 3 shows the emission of fungi when excited with UV LED. Just as in Figure 2, the spectra of different fungi are presented in two figures (Figures 3a and 3b).

Spectroscopic results show that fluorescence emissions induced by UV LEDs (Figure 3) are quite similar from the ones obtained with Wood's lamp (Figure 2). 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 fluorescence, although a careful spectroscopic analysis of the fluorescence shows distinct features on the detected emissions. In Table 1, fluorescence characteristics of fungi are presented. The peak wavelength (λ_{max}) and the bandwidth ($\Delta\lambda$) of the measured fluorescences are listed. The differences found on the fluorescence characteristics of some fungi on the first and second attempts probably result from the collection of growth media fluorescence.

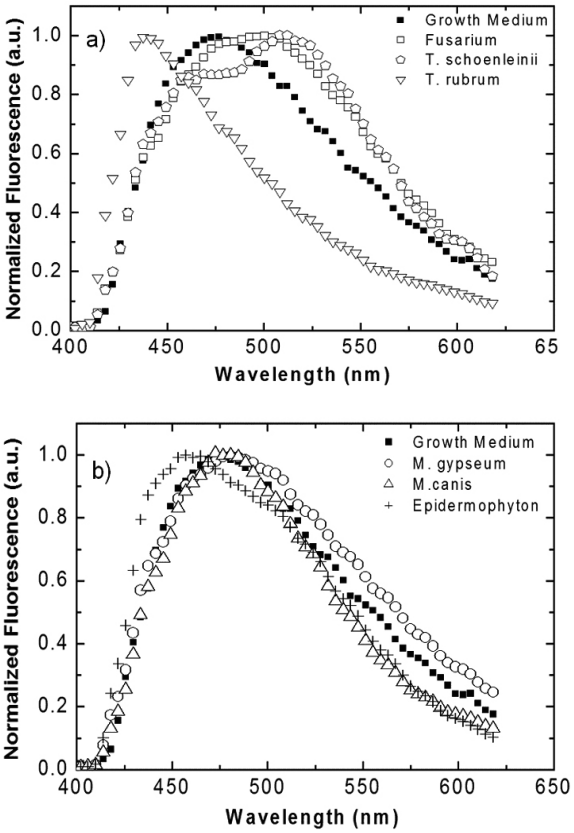

Figure 2. Fungal emission after UV lamp excitation (21 days after inoculation).

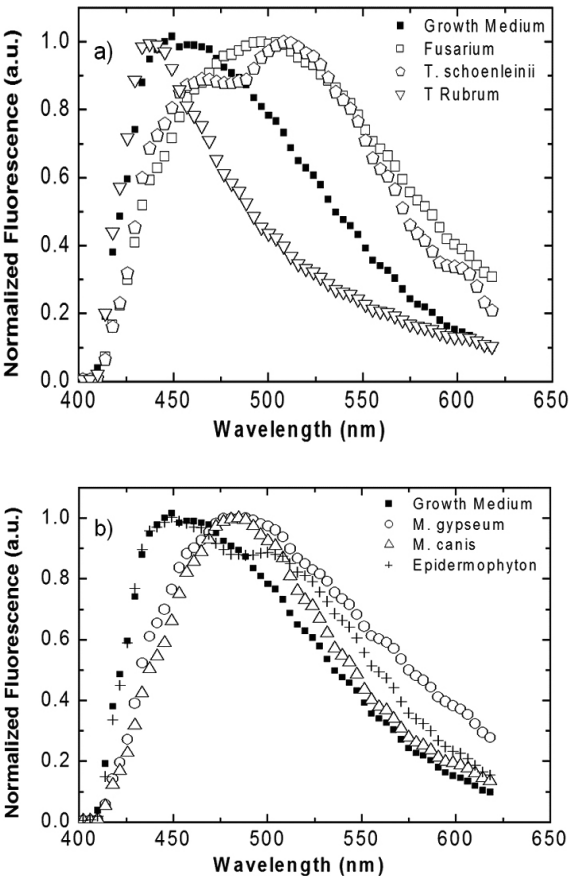

Figure 3. Fungal emission after UV LED excitation (21 days after inoculation).

Fungal emission spectra obtained using the narrow band UV Laser excitation source are presented in Figure 4. UV Laser induced fluorescence present three peaks, resulting from specific energy decaying channels. The graphics in Figures 4a and 3b 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 relative intensity defined as $(I_{\lambda1}-I_{\lambda2})/(I_{\lambda1}+I_{\lambda2})$, where $I_{\lambda1}$ and $I_{\lambda2}$ are the intensity of the fluorescence at 417 nm and 505 nm respectively,

Table 1. Fungal fluorescence spectral characteristics 21 days after inoculation (lamp and LED excitation).

	1st experiment				2nd experiment			
	LED		lamp		LED		lamp	
Fungi	λ_{max} (nm)	$\Delta\lambda$ (nm)	λ_{max} (nm)	$\Delta\lambda$ (nm)	λ_{max} (nm)	$\Delta\lambda$ (nm)	λ_{max} (nm)	$\Delta\lambda$ (nm)
T. rubrum	437 ± 5	72 ± 7	438 ± 5	73 ± 7	440 ± 5	68 ± 7	449 ± 5	75 ± 7
Fusarium	495 ± 5	150 ± 7	495 ± 5	140 ± 7	479 ± 5	164 ± 7	483 ± 5	150 ± 7
Schoenleinii	511 ± 5	141 ± 7	510 ± 5	138 ± 7	511 ± 5	141 ± 7	512 ± 5	143 ± 7
M. gypseum	482 ± 5	143 ± 7	480 ± 5	138 ± 7	–	–	–	–
M. canis	482 ± 5	108 ± 7	478 ± 5	107 ± 7	485 ± 5	149 ± 7	480 ± 5	116 ± 7
Epidermophyton	450 ± 5	130 ± 7	456 ± 5	121 ± 7	446 ± 5	119 ± 7	456 ± 5	126 ± 7
Growth medium	449 ± 5	108 ± 7	474 ± 5	120 ± 7	455 ± 5	126 ± 7	474 ± 5	123 ± 7

obtained in Figure 4. The results in Table 2 indicate that a spectroscopic analysis of UV Laser induced fluorescence can be explored on the identification of fungi.

The growth medium fluorescence upon UV irradiation is also shown in Figures 2, 3 and 4. It is important to observe that the fluorescence collected from some fungi is quite similar to the emission spectrum of the growth medium. We also noticed that growth mediums prepared (obtained) at different days may present slight different fluorescent spectra. Autofluorescence of the growth medium is been considered as a background under the fungi emission. *M. canis, M. gypseum* and *Fusarium* present a faint emission, and their characteristics (showed in Tables 1 and 2) can be misleading due to the excitation and collection of the growth medium fluorescence.

Table 2. Fungal fluorescence spectral characteristics 21 days after inoculation (Laser excitation).

Table 2	$(I_{\lambda 1}-I_{\lambda 2})/(I_{\lambda 1}+I_{\lambda 2})$	
Fungi	1st experiment	2nd experiment
T. rubrum	0.55	0.54
Fusarium	-0.09	-0.13
Schoenleinii	-0.13	-0.13
M. gypseum	0.25	–
M. canis	-0.10	-0.08
Epidermophyton	0.28	0.26
Growth medium	0.00	0.00

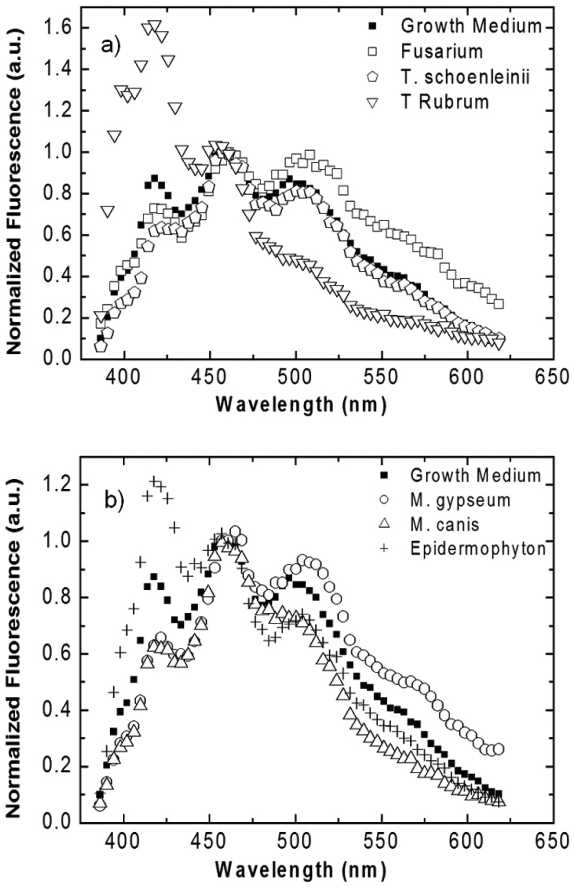

Figure 4. Fungal emission after UV Laser excitation (21 days after inoculation).

Fluorescence decay times were measured for all samples excited with Laser and LED light sources, and found to be smaller than 10 ns, the temporal resolution of our setup. These small decay times were expected for large molecules (McHale, 1998), particularly within a complex environment.

Discussion

We showed that different UV light sources can be used to induce fluorescence in several fungi. Different features from each fungal fluorescence (Table 1) do not change with the use of Wood's lamp or UV LEDs. Results of two attempts (carried out one month apart) indicate that the bandwidth and the peak wavelength of the fluorescence can be explored on fungi characterization. In spite being an expensive UV light source, UV Lasers can also induce fluorescence in fungi and it can be used in fungal identification. Different optical techniques exploring Lasers (Jarvis and Goodacre, 2004; Naumann, 2000), as Raman spectroscopy, can be explored in microbial diagnosis.

Moreover, we showed that LEDs can be applied in fungal identification, just as Wood's lamps in clinical diagnoses of fungal infection. Some limitation of the clinical "Wood's lamp" technique could be overcome by applying a careful spectroscopic analysis. Until recently UV LEDs were too expensive to be used on most lighting applications, but the price of semiconductor devices has plummeted over the past decade making LEDs a more cost-effective lighting option for a wide range of applications. While they may be more expensive than incandescent lights up-front, their lower long term cost can make them a better choice. In the future, LEDs will play an even bigger role in the biological and medical field.

Acknowledgements

This work had the financial support of the Brazilian agencies CNPq, FACEPE and CAPES. Cid B. de Araujo is acknowledged for sharing the laser and other equipment used in the experiments. We are grateful to M.F.S Oliveira for expert help in sample preparation.

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