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Evaluation of optical redox ratio in *Candida albicans* cells exposed to photobiomodulation

Tamara S. Adjimann[1,2], Thaila Quatrini Corrêa[1], Fernanda Alves[1], Sebastião Pratavieira[1*]

[1]University of São Paulo, São Carlos Institute of Physics, PO Box 369, 13560-970, São Carlos, SP, Brazil

[2]Current address: Instituto de Fisiología, Biología Molecular y Neurociencias (IFIBYNE), CONICET, Universidad de Buenos Aires, Buenos Aires, Argentina

ABSTRACT

Photobiomodulation (PBM) is the use of light in a specific wavelength and irradiance to induce some process in the cells such as death or division. One possible way to follow the light effects is through the optical redox ratio (ORR). ORR is an established microscope optical technique that uses the endogenous fluorescence of NADH (reduced form of nicotinamide adenine dinucleotide) and FAD (flavin adenine dinucleotide) to verify the metabolic route of a cell. The purpose of this study was to measure the effect of light on the ORR of *Candida albicans* cells, growing in aerobic and anaerobic conditions. Standardized *C. albicans* suspensions were adjusted in a spectrophotometer to 10^7 colony forming units per milliliter (CFU/mL) (540 nm) after growing 12 or 24 hours under aerobic and anaerobic conditions. The anaerobic state was achieved using an airtight jar with a candle inside. Then, the PBM was performed using a LED device emitting at 450 nm with an irradiance of 44 mW/cm^2 and a total fluence rate of 200 J/cm^2. The ORR was evaluated through confocal laser microscopy using laser excitation of 755 nm (NADH excitation) or 860 nm (FAD excitation), and images were acquired in the channel mode of the microscope with 440 - 480 nm (NADH fluorescence) or 500 - 550 nm (FAD fluorescence) wavelength range, respectively. The ORR images were created by computing pixel-wise ratios of FAD/(NADH+FAD) fluorescence. Cell viability was quantified by CFU/mL assay. According to the viability test, the anaerobic suspensions (12 and 24 h) showed lower growth in comparison with the aerobic suspensions (12 and 24 h). The ORR is statistically the same in aerobic and anaerobic conditions, and lower after the illumination. In conclusion, our preliminary study demonstrated that it is possible to measure the ORR in *C. albicans* cells and the effects of PBM in these cells.

Keywords: photobiomodulation; optical redox ratio; NADH, FAD, aerobic, anaerobic, *Candida albicans*

*prata@ifsc.usp.br; http://cepof.ifsc.usp.br/

1. INTRODUCTION

Candida is a genus of Fungi that includes more than a 150 different species. Although most of the *Candida spp.* are innocuous commensals and ordinary members of human´s flora, about 15 of them are known to cause a range of human diseases.[1,2] Among these species, the most predominant is *C. albicans* which can lead to a variety of infections, from superficial, mucosal and cutaneous, candidiasis, to systemic disease where the fungus reaches the bloodstream.[3,4] These pathological conditions usually arise in immunocompromised patients, where the harmless fungus becomes an opportunistic pathogen that begins to colonize the skin or the oral cavity.[5] *C. albicans* is among the leading causes of nosocomial bloodstream infections resulting in the decrease of around 40% of the severely immunocompromised patients.[6]

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The most common treatment for *Candida* infections are antifungal agents; however, there is evidence of increasing resistance to these products.[7] Therefore, the development of alternative therapies is of great importance.

Photobiomodulation (PBM) has been pointed out in several works as a possible treatment for *C. albicans* infections that does not involve antifungals.[8,9] PBM is based on the exposure of cells to a low power light source, generally a laser or an LED, in order to obtain the desired effect. Although PBM is sometimes used to induce cell proliferation, it can also lead to cell death.[10] The effect of the PBM depends on many parameters; some of the most important are the wavelength, the fluence and the irradiance.[11] When intending to treat a bacterial or fungal infection, the aim is to inhibit the proliferation of the pathogenic cells while not damaging the patient's cells. Research has shown that blue light, from 400 nm to 470 nm, can reduce pathogenic microbes without having detrimental effects on host cells.[12] PBM´s mechanism of action has not been completely elucidated yet. However, it is believed that the effects of blue light, at least in bacteria, are oxygen-dependent and due to the absorption by the Soret band of the porphyrins (around 405-420 nm), which results in the production of ROS (Reactive Oxygen Species), especially singlet oxygen, leading to oxidative stress and cell death.[13–15]A secondary mechanism has been proposed for longer wavelength blue light (440-490 nm) that involves light absorption by flavin molecules and their photoexcitation.[16]

Because of the way PBM is thought to work, it could be possible that the treatment with light induces changes in the metabolic pathway of the target cells. Also, as the mechanism is thought to be oxygen-dependent, differences could be expected between cells grown in anaerobic or aerobic conditions. *C. albicans* possess a classical respiratory chain consisting of Complexes I–IV, as well as a cyanide-insensitive alternative oxidase.[17,18] Moreover, there is evidence of a third "parallel" oxidative pathway that represents around 10% of total respiration capacity.[18,19] *C. albicans* has been described as a yeast with poor fermentation, meaning it highly relies upon oxidative phosphorylation for ATP production.[20] Therefore, respiratory function is critical in *C. albicans* growth and virulence. Nonetheless, the yeast can grow both in aerobic and anaerobic conditions.[21]

A useful non-invasive and dye-free method to assess the metabolic state of a group of cells is to measure the Optical Redox Ratio (ORR) by fluorescence microscopy. The technique is based in the detection of the endogenous fluorophores NADH (reduced form of nicotinamide adenine dinucleotide) and FAD (flavin adenine dinucleotide), which act as electron carriers in metabolic processes such as glycolysis and oxidative phosphorylation.[22] Measuring the fluorescence ratio of FAD/(FAD+NADH), it is possible to detect changes in the cell rate of these metabolic processes and consequently the cell redox state.[23] During oxidative phosphorylation, fluorescent FAD is generated from non-fluorescent $FADH_2$ and fluorescent NADH is converted to non-fluorescent NAD+. Therefore, an increase in the ORR occurs. An absence of oxygen or an increase in glycolysis is associated with the presence of a higher amount of NADH, meaning a decrease in the ORR.[23,24] NADH and FAD have an excitation maximum at 350 nm and 450 nm respectively, its emission maximum is at 460 nm for NADH and at 535 nm for FAD.[25] A good method to measure the ORR both *in vivo* and *in vitro* is using two-photon microscopy. In this technique, the excitation is due to the simultaneous absorption of two photons of twice the wavelength and half the energy of the required with one single photon.[24] With this technique, it is possible to excite NADH and FAD with near-infrared light (NIR), which is relatively benign, instead of potentially harmful ultraviolet (UV) and near-UV light.[24,25]

The purpose of this study was to measure the effect of light on the ORR of *C. albicans* cells, growing in aerobic and anaerobic conditions. If the ORR changes accordingly to the PBM effects, it could be an excellent way to assess the effectiveness of a treatment.

2. MATERIALS AND METHODS

2.1 Strain and growth conditions

C. albicans strain from American Type Culture Collection (ATCC number 90028), was reactivated in Sabouraud Dextrose Agar (Himedia® – HiMedia Laboratories Pvt. Limited, Mumbai, India) plate in incubator at 37 °C for 48 hours. After incubation, 5–10 colonies were resuspended in a tube with 10 mL of Tryptic Soy Broth (TSB) and the fungus was incubated at 37 °C for 16 hours. After this period, *Candida* suspension was standardized at optical density of 0.6 arbitrary units (a.u.), determined in spectrophotometer (Varian Cary® 50 UV-Vis Spectrophotometer - Agilent, Santa Clara, California, USA),

equivalent to 10^7 cells/mL. Then, the standardized suspension was grown for 12 or 24 h at 37 ºC. Some groups were growth in aerobic conditions inside a shaker (75 RPM) and others were growth in hypoxic conditions. The hypoxic condition was achieved using a hermetic jar where the samples were placed alongside a candle that was lit before closing the jar tightly. The fire consumed the oxygen, which led to the state of hypoxia. After growth, the cells were collected by centrifugation, washed three times and suspended in 100 μL of phosphate-buffered saline (PBS) in order to be observed at the confocal microscope.

2.2 Irradiation protocol

In order to evaluate the changes in the redox ratio under stress, some groups were illuminated using a custom made LED array device emitting at 450 nm with an irradiance of 44 mW/cm^2 and a total fluence rate of 200 J/cm^2 , these treatment has been proven previously to induce cell death.[26] The samples were observed at the confocal microscope immediately after illumination.

2.3 Microscopy and calculation of Redox Ratio

Images were performed on an inverted fluorescence confocal microscope (Zeiss - LSM780, Zeiss, Jena, Germany) equipped with a Ti:Sapphire tunable laser source (Chameleon Vision II, Coherent Inc., Palo Alto, CA, USA). The laser excitation source was tuned to 755 nm (NADH fluorescence) or 860 nm (FAD fluorescence), and images were acquired in the channel mode of the microscope with 440 - 480 nm (NADH) or 500 - 550 nm (FAD) wavelength range, respectively. Images (1024 x 1024 pixels; 8-bit depth; 212.34 μm x 212.34 μm) were acquired using a 20x objective (NA = 0.8). All images were acquired using Zen 2010 software (Zeiss, Jena, Germany). Image analysis was performed using MATLAB (MathWorks, Natick, USA) and the redox images were created by computing pixel-wise ratios of FAD/(NADH+FAD) fluorescence.

2.4 Cell viability

Cell viability was quantified by CFU/mL assay. Immediately after irradiation, 100 μL of each group were transferred into microtubes holding 900 μL of PBS to carry out the serial dilution until 10^{-5}. Aliquots of 25 μL of all dilutions of groups were uniformly spread to Petri dishes with Brain Heart Infusion (BHI) Agar medium in duplicate. Plates were maintained at 37 °C for 48 h to carry out the counts of CFU.

2.5 Statistical analysis

The data were plotted using boxplot with a whisker of 1-99 and were analyzed using the commercially available software Origin 2018 (Origin Lab., USA). One-way analysis of variance (ANOVA) was used among the two categories “aerobic” and “anaerobic” conditions and “Control” and “PBM” for the ORR measurements. Differences were considered as statistically significant at $p<0.05$. The black bars placed above indicate statistical significance.

3. RESULTS AND DISCUSSION

Figure 1 shows the emission of NADH (false color blue) and FAD (false color green) from the planktonic form of *Candida albicans*. The fluorescence emission is observed along all the cell indicating the presence of these important molecules.

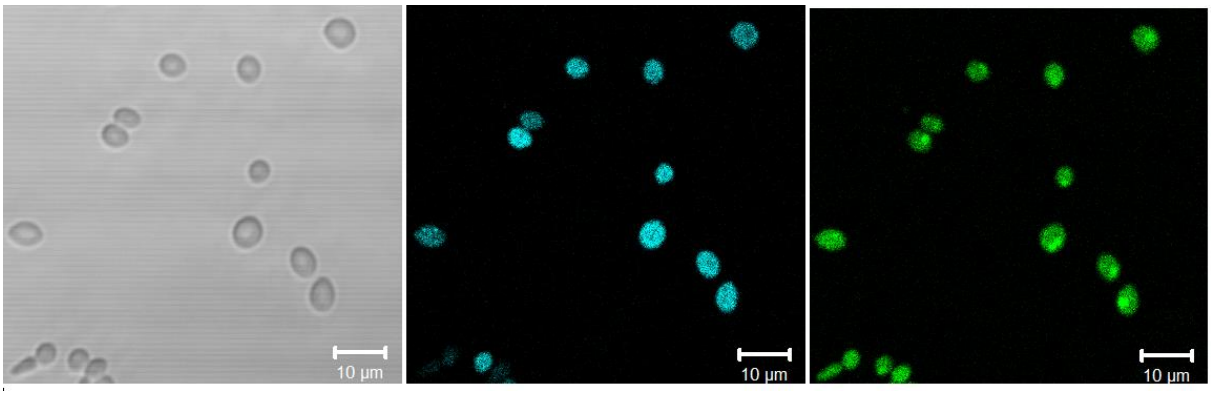
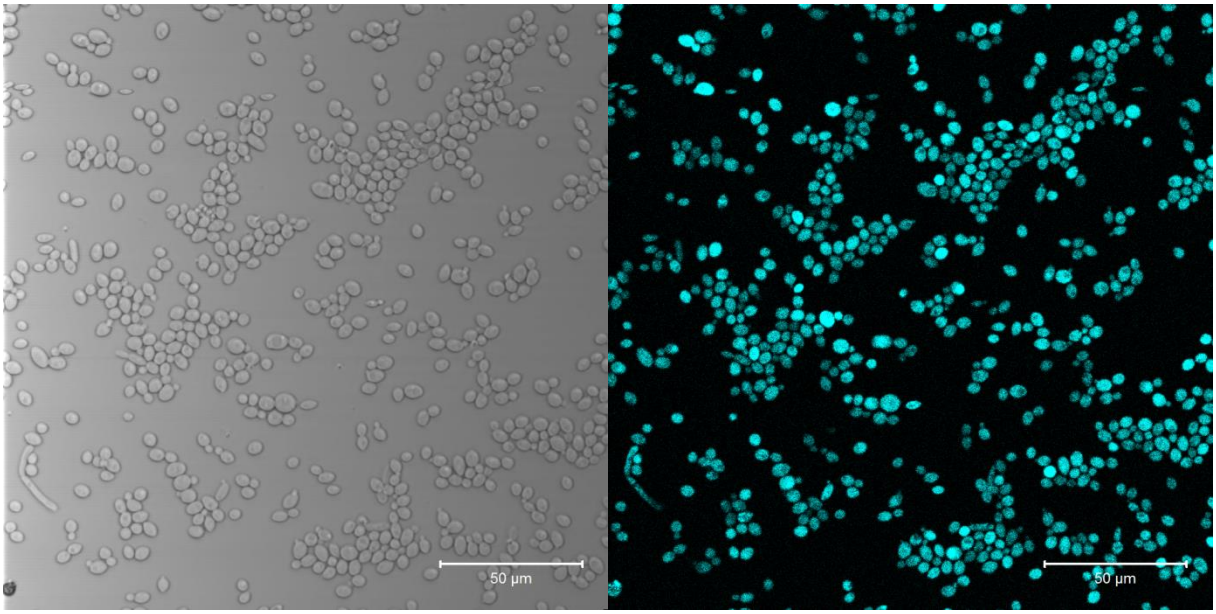


Figure 1: a) bright field image; b) fluorescence emission of NADH; c) fluorescence emission of FAD.

In figure 2 is presented the images of bright field, NADH, FAD and the ORR of the *Candida albicans* cells. The ORR is present in false color with the intensity according to the color bar intensity.



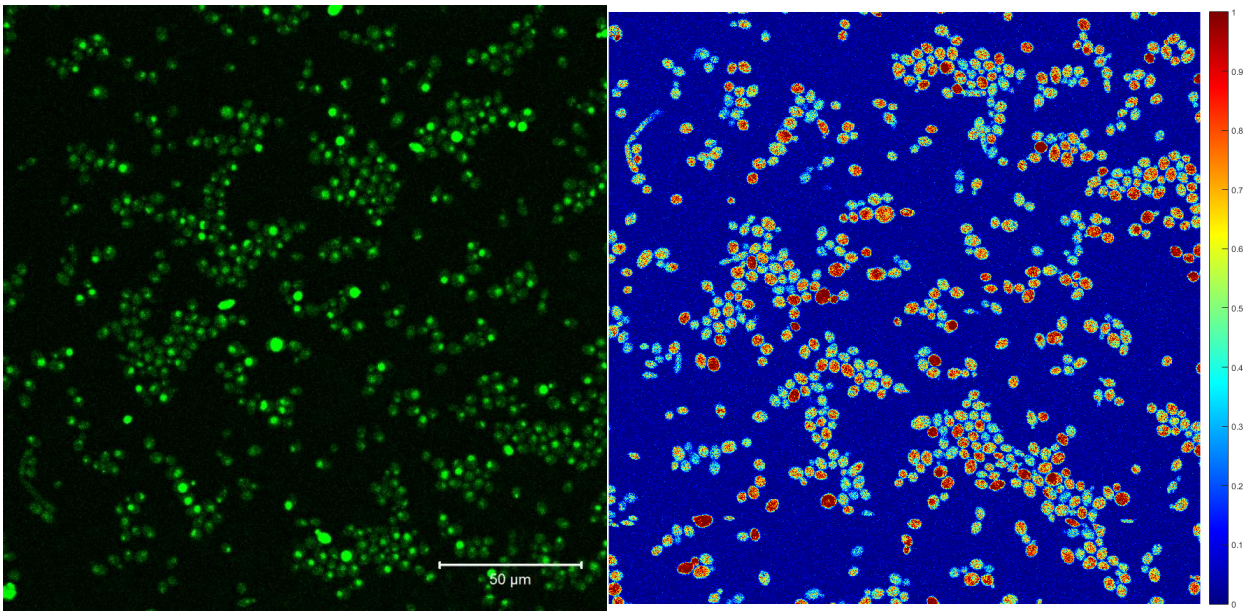


Figure 2: a) bright field image; b) fluorescence emission of NADH; c) fluorescence emission of FAD; d) redox ratio image.

The results of the ORR calculated for the experimental groups are shown in Figure 3. The growth in an aerobic or anaerobic condition did not affect the ORR for 12 h or 24 h groups. According to the viability test (CFU/mL assay), the anaerobic suspensions (12 and 24 h) showed lower growth in comparison with the aerobic suspensions (12 and 24 h).

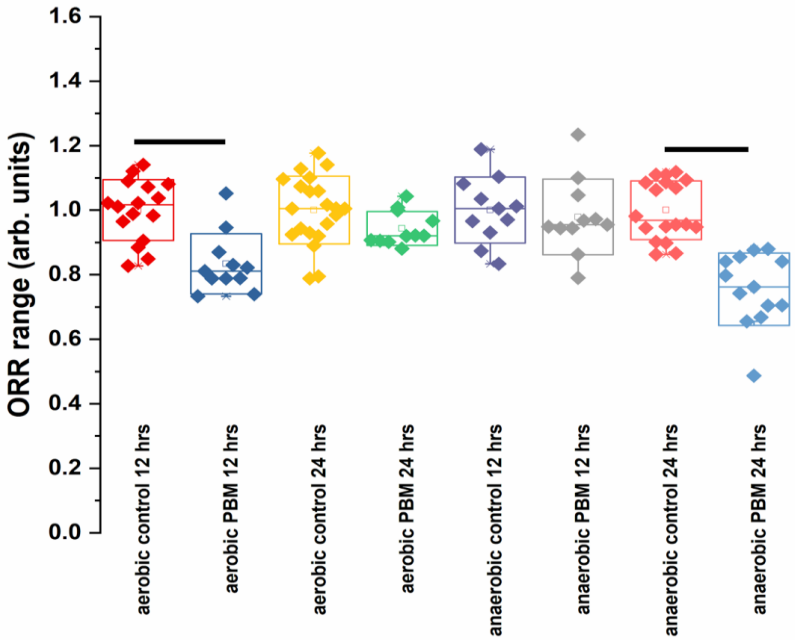


Figure 3: Redox ratio comparison between the analyzed conditions. The black bars placed above indicate statistical significance.

4. CONCLUSIONS

To the best of our knowledge, for the first time was measured the optical redox ratio of a fungus. The *candida albicans* has an intense fluorescence of NADH and FAD. The growth conditions in an anaerobic and anaerobic environment did not show a significant alteration on the ORR. Whereas the illumination protocol induces a reduction in the ORR.

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