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Time-resolved fluorescence lifetime for cutaneous melanoma detection

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Abstract: Melanoma is the most aggressive skin cancer type. It is characterized by pigmented lesions with high tissue invasion and metastatic potential. The early detection of melanoma is extremely important to improve patient prognosis and survival rate, since it can progress to the deadly metastatic stage. Presently, the melanoma diagnosis is based on the clinical analysis of the macroscopic lesion characteristics such as shape, color, borders following the ABCD rules. The aim of this study is to evaluate the time-resolved fluorescence lifetime of NADH and FAD molecules to detect cutaneous melanoma in an experimental in vivo model. Forty-two lesions were analyzed and the data was classified using linear discriminant analysis, a sensitivity of 99.4%, specificity of 97.4% and accuracy of 98.4% were achieved. These results show the potential of this fluorescence spectroscopy for melanoma detection.

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1. Introduction

Annually, 2-3 millions non-melanoma and around 132,000 melanoma skin cancers occur globally and, unfortunately, these numbers are increasing, according to the World Health Organization. Surgical resection is only effective for initial lesions, and its recurrence can vary from 7 to 51%. In Brazil, approximately 6,000 lesions are diagnosed per year, and melanoma is responsible for 80-85% of all deaths caused by skin cancer. Melanoma is the most aggressive skin cancer type, which is characterized by pigmented lesions with high metastatic potential. The early detection of melanoma is extremely important to improve patient prognosis and decrease mortality rate. Presently, the melanoma diagnosis is based on the clinical analysis of the macroscopic lesion characteristics such as shape, color, borders following the ABCD rule. The ABCD rule states for: A (asymmetry), B (border irregularity), color (non-homogenous color distribution), and D (diameter greater than 6 mm). The lesion progression and site location are also considered for clinical diagnosis [1].

As soon as the clinical examination indicates a potential melanoma, an excision is performed and then, the histology is evaluated. If melanoma cells are detected in the border of the lesion, another excision is performed with a larger resection margin. Chemotherapy and radiotherapy are also indicated for the cases of possible metastatic stage [2]. Anyway, early detection of melanoma is highly dependent on the clinician training and expertise on recognizing initial melanoma signs. Optical techniques for diagnosis have been already used for melanoma detection such as epiluminescence microscopy, reflectance spectrophotometry, fluorescence spectroscopy, fluorescence imaging and optical coherence tomography [3, 4].

Melanoma cells are characterized by high proliferative and metabolic rates, but the most common variation between normal and cancer cells is the high rate of aerobic glycolysis or the Warburg effect. The Warburg effect describes the cancer respiratory pathway that, when in the presence of oxygen, shows a high rate of glycolysis, followed by the lact acid fermentation in citosol, in contrast to the normal cells that present a lower glycolysis rate, followed by oxidation of pyruvate in mitochondria. In normal tissue, the glucose metabolism pathway is described by the Pasteur Effect, which determines the metabolism pathway with plentiful and limited oxygen [5, 6]. NADH (nicotinamide adenine dinucleotide) and FAD (flavin adenine dinucleotid) are the primary electron acceptor and donor, respectively, in oxidative phosphorylation, the most efficient means of energy production in cells. Changes in the NADH/FAD rates and their relative amounts of free and protein-bound states depend on the glycolysis and oxidative phosphorylation ratio. This suggests that tissues with different metabolic rates can be discriminated by comparing short and long lifetime fluorescence decays of NADH (the average lifetimes for free and protein-bound NADH are approximately 0.3 ns and 2 ns, respectively) and FAD (for free FAD is around 5 ns and, for protein-bound FAD, approximately 1 ns) and the relative amount of free and protein-bound states [7]. Although NADH and FAD are the mainly molecules used as biomarkers for tumor metabolism, the fluorescence lifetime technique reveals changes in the tumor microenvironment, and is influenced by others fluorophores present in tissue. The individual
fluorescence analysis of specific molecules in tissue measurements is a complex task, since the overall spectrum presents the contribution of several biomolecules, including absorbers and scatterers, and is modified by attenuation coefficient at the visible spectrum. Anyway, the detection of these overall changes in tumor microenvironment is an important factor for tumor and margin evaluation.

Fluorescence lifetime analysis has been presented as a sensitive technique for tissue characterization for diagnosis purposes. The fluorescence lifetime can discriminate free and protein-bound components of fluorophores. One of the main advantages of the fluorescence techniques is the in situ and non-destructive evaluation of tissues with fast response. This makes the fluorescence lifetime technique quite suitable for clinical applications. Targeting NADH and FAD from cutaneous melanoma lesions may present diagnostic information. The aim of the present study is to evaluate the efficacy of the time-resolved fluorescence spectroscopy on the diagnostics of the induced cutaneous melanoma in an animal model.

2. Materials and methods

Cell line: The B16F10 murine melanoma cells (Rio de Janeiro Cell Bank, Rio de Janeiro, Brazil) were cultured in DMEM medium supplemented with 10% bovine fetal serum and 1% penicillin-streptomycin solution under 5% CO\textsubscript{2} at 37°C.

Animals: Forty-two nude balb/c mice (Centro de Bioterismo - Universidade de São Paulo – São Paulo, Brasil) were housed in micro-isolator cages, with food and water ad libitum.

Experimental melanoma: The animal was anesthetized using 2% isofluorane mask. An intradermal injection of 10^7 B16F10 cells in 100 µL of PBS was performed into the right and left animal flank regions. The mice were examined daily to monitor any changes in systemic conditions as loss of weight or signs of metastasis. This study was approved by the Ethical Committee of the Universidade Federal de São Carlos – São Carlos/Brazil.

Characterization of optical properties of the experimental melanoma: The light distribution in the induced tumor was evaluated by reflectance, fluorescence and transmission spectroscopy measurements. For the reflectance and fluorescence measurements, the optical fiber coupled to 378nm or 445nm laser was positioned on the top of the tumor, and the reflectance was radially collected scanning the total lesion surface, or until the signal absence, in steps of 0.1mm. Then, the animal was euthanized and the tumor was removed for transmittance evaluation. For experimental purposes, an optical fiber was positioned at the top of the lesion, and the collect fiber was positioned at the tumor base to collect the transmission light. The diffuse and transmitted reflectance spectra were plotted and the total attenuation coefficients were determined based on the dimensional lesion characteristics. Fluorescence spectra were also plotted and the maximum distance at the lesion surface from the excitation site was determined. Based on these measurements, an estimated interrogated tissue volume for this experimental melanoma was defined.

Fluorescence system: The time-resolved fluorescence spectroscopy system is composed of two diode lasers, one emitting at 378 nm and another at 445 nm (BDL-375-SMC and BDL-445-SMC, Becker and Hickl, Berlin, Germany). The 378 nm is used to excite NADH, and the 445 nm to excite FAD. Both lasers have a repetition rate of 20, 50, 80 MHz and a temporal pulse duration of 50-100 ps. A bifurcated fiber (Ocean Optics, Dunedin, Florida, USA) was used to deliver the light to the tissue and to collect the tissue fluorescence. A bandpass filter at (440 ± 40) nm (Semrock, Rochester, New York, USA) was used with 378 nm excitation, and a bandpass filter at (514 ± 30) nm (Semrock, Rochester, New York, USA), for 445 nm excitation. The fluorescence signal was detected using a high-speed hybrid PMT detector (HPM-100-50, Becker and Hickl, Berlin, Germany). The data were collected and processed using the TCSPC Software (Becker and Hickl, Berlin, Germany). The impulse response function (IRF) of the system is about 230 ps.

Fluorescence lifetime measurements: Lesions of 2 up to 30 mm in diameter were interrogated. The mice were anaesthetized with 2% isofluorane in oxygen using a mask.
optical fiber was positioned at the surface of the experimental melanoma lesion or on the normal skin and the fluorescence measurements were collected. At least three measurements were taken from each lesion with both excitation lasers. Each flank region where the melanoma cells were inoculated was monitored daily, and the measurements were performed with lesions size between 2 and 15mm. A total of 42 lesions were investigated.

**Data processing:** The raw data of the photon counting with time resolution was processed using the SPCImage Software (Becker and Hickl, Berlin, Germany). SPCImage software provides an “estimation” of the IRF by calculating the first derivative of the rising part of the fluorescence lifetime spectrum and, then, it calculates a function model for the measured intensity by the convolution of the IRF and the exponential decay fitting. So, the decay curve of the model function was fitted by a bi-exponential function:

$$F(t) = a_1 e^{-\frac{t}{\tau_1}} + a_2 e^{-\frac{t}{\tau_2}},$$

using the average fluorescence lifetime values $\tau_1$ and $\tau_2$, corresponding to short and long lifetime components, respectively, and relative coefficients $a_1$ and $a_2$, where $a_1 + a_2 = 100$.

In order to compare the collected data from normal skin and cutaneous melanoma, scatter plots and box plots graphs were constructed using Origin 9.0 (OriginLab. Corporation, Northampton, MA, USA) using $a_1$, $a_2$, $\tau_1$, and $\tau_2$ parameters of each measurement. This comparison allowed a better discrimination for measurements using 378 nm or 445 nm excitation wavelengths.

**Statistical analysis:** It was used unpaired t test. Exact p values were computed, all p values are two-sided, and a $p < 0.001$ was considered statistically significant.

**Data analysis:** a classification was performed with the linear discrimination analysis of MATLAB software (version R2012a, Mathworks, Natick, MA, USA) to determine the combination of predictor variables that accounts for most of the differences in the behaviors of the normal skin and cutaneous melanoma groups. The classification algorithms were then evaluated based on sensitivity, specificity and accuracy values. For diagnostics classification, histopathology of HE slides was used as gold standard.

### 3. Results

**Experimental melanoma:** the induced cutaneous lesion shows a fast progression characterized by dark pigmentation and metastatic potential. Twenty-four hours after the injection it is possible to observe a small pigmented lesion with an average of 2-3mm in diameter. At 48 hours after the melanoma induction, the lesions reach up to 1cm of diameter. After 7 days, the tumor shows a diameter of 4-5cm. During this development, no significant changes were observed in the animal overall conditions, as loss of body weight or changes in behavior. Ten days after the induction, metastasis can be observed. In the first 48 hours of development, some lesions present a thin layer of normal epidermis over the tumor. It constitutes a limitation for optical measurements such as fluorescence lifetime, especially with ultraviolet-violet excitation, since the detected signal has also the contribution of normal tissue presented. The induced lesion rises at the surface until approximately 72 hours, becoming similar to a spontaneous lesion. After 10 days it starts to invade deeper tissues, reaching muscle and spreading to all the body. Even before invasion, the lesion area observed on the skin surface was smaller than that observed in dermis deeper layers. During this period, angiogenesis is also observed, but this is not sufficient to feed the whole tumor mass and the lesion center become necrotic. For experimental purposes, no measurements were performed in the necrosis area, due to the absence of lesion diagnostic information. In the histology one can observe melanoma cells surrounded by a collagen fiber and numerous blood vessels.

**Characterization of experimental melanoma optical properties:** the 378nm laser reflectance measurements showed that the light is completely absorbed by the tissue and so, no reflectance was observed at the surface. On the other hand, it was possible to observe the
tissue fluorescence as a function of the radial distance. The decays were fitted and the attenuation coefficient determined. Then, an interrogated volume was estimated in about 0.079 ± 0.009 mm$^3$. For the 445nm laser, due to its low efficiency for tissue excitation, it was not possible to detect the fluorescence signal. However, the reflectance measurements were enough to estimate the probed volume to 0.017 ± 0.002 mm$^3$. Due to the lesion thickness between 2.4mm and 4mm, transmittance signal was not detected for none excitation lasers.

*Time-resolved fluorescence lifetime spectra:* the fluorescence lifetime is sensitive to fluorophore microenvironment and provides a method for discriminating free and protein-bound components of NADH and FAD. The NADH molecules show a short lifetime component when it is free and a longer lifetime component when it is protein-bound. For FAD molecules, short lifetime component is present for protein-bound and longer lifetime component for its free state. These molecules states are related to aerobic and anaerobic processes: oxidative phosphorylation and glycolysis. In Fig. 1 one can observe the decay curve for (mainly) NADH and FAD molecules in normal skin and melanoma.

**Fig. 1.** Representative fluorescence decay profiles of NADH (A) and FAD (B) molecules in normal skin and melanoma.

Despite the fact that the system excites targeting NADH and FAD molecules, it is complex to evaluated individually the contribution of these molecules in the in vivo fluorescence spectrum. Other biomolecules also contribute for the collected spectrum such as melanin, collagen, elastin and keratin.

Data processing: The results obtained for the 378nm excitation is shown in Fig. 2. One can observe that for the $a_1$ and $a_2$ parameters there were no significant difference between normal skin and cutaneous melanoma (Fig. 2(A) and 2(B)). It was not observed in the melanoma, probably due to the thin normal epidermis layer present over the experimental lesion. In addition to its contribution to the collected fluorescence signal, this thin skin layer also reduces the laser penetration and resulted excitation of the target tissue.

On the other hand, the parameters $\tau_1$ and $\tau_2$ that refers to the average lifetime fluorescence values showed a statistical significant discrimination between normal skin and melanoma ($p < 0.001$)(Fig. 2(C) and 2(D)). Both, short and long lifetime components, increased in melanoma when compared to normal skin. If the results agreed with the Warburg effect, the melanoma fluorescence lifetime would be diminished due to the presence of higher concentration of free NADH molecules when compared to protein-bound molecules. However, this was not observed. A hypothesis to these results is that since we interrogated the melanoma lesions at different development stages, the specific Warburg effect could not be observed at the average data. Furthermore, others molecules such as collagen and melanin may contribute to this change in the fluorescence lifetime observed. This data was also fitted for three
exponentials, but the parameters obtained did not improve our results. Anyway, these measurements showed a significant discrimination between normal skin and melanoma.

Figure 2. Boxplot for the parameters obtained in the data analysis for 378 nm excitation: $a_1$, $a_2$, $\tau_1$, and $\tau_2$, respectively. Significant differences ($P < 0.001$) exist between normal vs. melanoma for the parameters $\tau_1$ and $\tau_2$ (*).

Figure 3 shows the fluorescence lifetime parameters obtained for FAD excitation at 445nm. The parameters $a_1$ and $a_2$ represent the relative quantities of FAD molecules on both states, protein-bound and free, respectively. Figure 3(A) and 3(B) show that melanoma has more FAD in protein-bound state than normal skin. The opposite is also true: normal skin has more free FAD molecules than melanoma. If the glycolysis and oxidative phosphorylation ratio characteristic of melanoma were observed, melanoma would present higher amount of free FAD than in normal tissue. However, looking for the normal skin, the relative quantities of free and protein-bound FAD agree with the aerobic metabolism. These results may be explained by the system used for the measurements. Although FAD is the mainly excited molecule, it is not the unique one.
The protein-bound FAD is part of the oxidative phosphorylation metabolism and its lifetime is smaller when compared to the free FAD state. Therefore, the short and long lifetime components describe the melanoma preference to glycolysis (Fig. 3(C) and 3(D)). These results agree with the melanoma metabolism changes described by Scott et al., 2011 [6]. Melanoma, even in the presence of oxygen, increases the energy generation by nonoxidative breakdown of glucose – glycolysis, instead of more energy-efficient oxidative respiration.

Data analysis: The data classification was performed using the parameters $\tau_1$ for 445 nm excitation and $\tau_2$ for 378 nm. These parameters were chosen based on the best results obtained in this study. The linear discriminant analysis was able to discriminate normal skin and melanoma with a sensitivity of 99.4%, specificity of 97.4% and accuracy of 98.4% (Fig. 4).
Fig. 4. Short lifetime component ($\tau_1$) of 445 nm excitation against long lifetime component ($\tau_2$) of 378 nm, the separating lines was performed using linear discrimination analysis.

The short lifetime component of FAD and long lifetime component of NADH provides information about the oxidative phosphorylation metabolism pathway. The aerobic respiration is present in both tissues following the Pasteur effect. Furthermore, it was an important parameter to distinguish normal skin and melanoma.

4. Discussion

Metabolism is an important parameter to evaluate the cell microenvironment. Nicotinamide adenine dinucleotide (NAD$^+/\text{NADH}$) is an important coenzyme involved in the energy metabolism of living cells. In the aerobic respiration, glucose is converted to lactate, NADH is oxidized to generate ATP through oxidative phosphorylation. NADH has a short and a long lifetime components, depending on whether it is in free or protein-bound state, respectively [6].

FAD is another coenzyme in living systems and is also present in two forms, referred to as protein-bound and free FAD. The protein-bound FAD state participates in oxidative phosphorylation and it is also important for tumor metabolism [6]. FAD also has, short and long lifetime components refer to protein-bound and free FAD state, respectively. Changes in the tumor’s microenvironment such as the oxygen viability, pH, and temperature modify the metabolism process, and may be detected by time-resolved fluorescence lifetime measurements.

Results obtained in experimental melanoma model with excitation at 378nm were able to distinguish normal skin and tumor for both lifetime components. However, parameters $a_1$ and $a_2$ were not able to distinguish normal skin and melanoma. These components are related to the amount of NADH molecules in the free and protein-bound states and the measurements may be compromised due to the experimental melanoma development. A thin normal epidermis can be observed overlaying the melanoma lesion in the first three days after the cells injection. This tissue layer probably contributes to the amount of NADH measurements modifying the parameters $a_1$ and $a_2$. It also influences the long and short lifetime components, but it is not visible due to the average lifetime obtained. Therefore, both lifetime components distinguished the normal skin and melanoma. These results do not agree with the Warburg Effect that describes the tumor metabolism preference to glycolysis (Scott et al., 2011). If it happens, melanoma would present lower fluorescence lifetimes due to the free NADH state lifetime contribution. There are many reasons that may explain this, such as experimental melanoma development, tumor pH and oxygen. Some endogenous fluorophores such as tryptophan moieties, collagenase-digestible and pepsin-digestible collagen, cross-links, elastin cross-links, reduced NADH, oxidized flavins, porphyrin and keratin may contribute to change the NADH fluorescence lifetime in melanoma. Several studies using fluorescence lifetime
presented different behaviors for normal and malignant tissues that are neither all in agreement with the Warburg effect. Skala et al. (2007) evaluated the fluorescence lifetime in precancerous epithelia and showed a decrease in the NADH lifetime when comparing normal to low and high grade precancerous [8].

The experiments performed with the 445nm excitation showed statistical significance for all components evaluated. Melanoma presented a higher amount of protein-bound state FAD in comparison to normal skin. It does not reveal the expected, wherein the protein-bound state FAD is related to the oxidative phosphorylation metabolism. Although the relative quantities of FAD did not agree with the Warburg effect, the opposite was observed for the lifetimes. The protein-bound FAD state has a short lifetime, while the free FAD has a long lifetime. Results showed that the melanoma has a longer lifetime, related to the presence of free FAD molecules. On the other hand, the normal skin showed a shorter lifetime, related to the protein-bound FAD. These results agree with the tumor preference to glycolysis. pH is another parameter that may influence the FAD lifetime. Islam et al. (2013) described that acidic pH shift the fluorescence lifetime to higher values, while basic pH shifts the fluorescence lifetime to smaller values [9]. This shift to higher lifetime values was observed in melanoma measurements, and it may be related to the pH effect once there are several studies describing the melanoma acidic characteristic [10,11]. Our results based on an averaged of the fluorescence lifetime measured at distinct melanoma development stages may not be specifically explained by the Warburg effect. The analysis of the NADH and FAD, free and protein-bound states, has not been completely elucidated during at each step of the tumor development. Tumor metabolic pathways is a complex process involving many factors such as cell type and proliferation rate, temperature, experimental model including the animal and others [12]. Beyond that, it is not possible to measure the fluorescence lifetime of only NADH and FAD molecules. They are the mainly molecules present in tissue, but collagen, melanin, keratin and others molecules can be find and definitely contribute to the fluorescence lifetime. Tumor metabolic stages and the amount of molecules present in tissue may be the responsible of changes in the decays.

Butte et al. (2005) described the potential use of time-resolved fluorescence spectroscopy as an adjunctive tool for the intraoperative rapid evaluation of meningioma diagnosis. They evaluated the technique on ex vivo tissue specimens from patients undergoing brain tumor surgery and the results showed a sensitivity higher than 89%, specificity of 100% and an accuracy of classification around 92% [13].

Marchesini et al. (2002) reviewed the most important optical systems for melanoma detection. Video camera showed specificity from 55 to 91%, sensitivity from 80 to 90%. The best results were obtained for infrared and RGB gradient color [3, 14, 15]. Spectrophotometry techniques showed specificity from 46 to 91% and sensitivity from 80 to 89%. The best results were obtained for lesion area and spectral curve analysis [16]. Elbaum et al. 2001 investigated the potential of the multispectral dermoscopy to diagnose melanoma. Sixty-three cutaneous melanoma and 224 non-melanoma lesions were evaluated. In this study, the infrared and wavelet data were evaluated and 85% specificity and 100% sensitivity were obtained [17].

Multiphoton laser tomography and fluorescence lifetime was also evaluated for melanoma [4]. This technique was able to distinguish cell morphology such as keranocytes and melanocytes, although this technique did not distinguish melanoma and nevus.

The main advantage of time-resolved fluorescence lifetime comparing with steady-state fluorescence spectroscopy is the possibility to obtain metabolic information that is not dependent on tissue absorption and scattering [8]. This advantage is highly important for melanoma. It is also important for margin detection during a surgery resection.

Using the time-domain fluorescence lifetime, our results showed a sensitivity of 99.4%, specificity of 97.4% and accuracy of 98.4% when it was used a linear classification. These results refer to 42 experimental melanoma lesions induced in balb/c nude mice using the cell
line B16F10. Though these results were obtained with an experimental melanoma, the values of sensitivity, specificity and accuracy were significant when compared to other techniques.

Despite this study reveals that the fluorescence lifetime technique was able to distinguish normal and melanoma tissue, its potential use for melanoma and margins diagnostics must be defined based also on the discrimination of melanoma and others pigmented lesions. The investigation of the efficacy of this technique in cutaneous pigmented lesions will be performed in patients. Anyway, the differentiation of normal skin and melanoma is very important for margin detection in surgery, once melanoma is an extremely aggressive tumor with high rates of morbidity and mortality when metastasized. The possibility of using a fast measurement to determine the tumor site resection may reduce the recurrence rate and then, improve the patient survival.

5. Conclusion

High rates of sensitivity, specificity, and accuracy of 99.4%, 97.4%, 98.4%, respectively, were obtained using time-domain fluorescence spectroscopy to detect cutaneous melanoma in an animal model. These results show the potential of lifetime fluorescence to aid in the cutaneous melanoma detection through a non-invasive technique.

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