Near-infrared autofluorescence spectroscopy and photobleaching detection of melanin-pigmented cutaneous neoplasia

V Mircheva¹,²*, L Zaharieva¹,², S Ilyov¹,², P Troyanova³, I Lihacova⁴, A Lihacovs⁴, I Bratchenko⁵, L Bratchenko⁵, Yu Khristoforova⁵, V Zakharov⁵, L Avramov¹ and E Borisova¹

¹ Institute of Electronics, Bulgarian Academy of Sciences, 72 Tsarigradsko Chaussee bld., 1784 Sofia, Bulgaria
² Faculty of Physics, St. Kliment Ohridski University of Sofia, 5 James Bourchier blvd., 1164 Sofia, Bulgaria
³ Tsaritsa Yoanna-ISUL University Hospital, 8 Byalo More str., 1527 Sofia, Bulgaria
⁴ Institute of Atomic Physics and Spectroscopy, 19 Raina blvd., Riga, Latvia
⁵ Department of Laser and Biotechnical Systems, Samara National Research University, 34, Moskovskoe Shosse blvd., 443086, Samara, Russian Federation

*E-mail: victoriamircheva@gmail.com

Abstract. In the current study, an excitation laser source emitting at 785 nm (100 mW, CW) was used to obtain ex vivo fluorescence of endogenous melanin in pigmented skin with benign, dysplastic and malignant cutaneous lesions. The samples were obtained after surgical removal during standard excision procedure and split for spectral analysis and histological verification. The samples of benign (BN-5) and dysplastic (DN-3) nevi and of malignant melanoma (MM-7) were used as representative for harmless/harmful cutaneous neoplasia with a similar melanin pigment ratio. Emission in the range 800 – 1100 nm was detected and compared using a USB4000 micro-spectrometer (Ocean Optics Inc., USA). The photobleaching dynamics was observed for the emission maxima at 825 nm for 10 minutes with a step of 20 s. These near-IR autofluorescence spectra may be assigned to a single fluorophore if appropriate excitation wavelength is applied, which simplifies the rapid analysis related to the appearance and concentration evaluation of a given type of endogenous fluorophore, as well as allows the evaluation of some parameters, such as photobleaching dynamics, as a diagnostic indicator assessing the tissue state. A diagnostic accuracy of 93.3% was achieved for MM lesions validation when the NIR fluorescence intensity and photobleaching rate values were used to discriminate between nevi and melanoma lesions.

1. Introduction

Many optical techniques have been applied recently in the clinical practice to obtain new data for different skin neoplasia. One of the approaches with high sensitivity is based on detecting the endogenous fluorescence of pathological alterations of cutaneous tissues, including the development of cancerous tissues. The fluorescence techniques provide information on the biochemical alterations and the morphological changes in the extracellular matrices of the tissues investigated [1-3]. The UV-VIS
autofluorescence diagnostics is based on detecting emission from amino acids, proteins, co-enzymes and vitamins, which have overlapping excitation and emission spectra in the ultraviolet and visible spectral ranges. This overlapping leads to some difficulties in the spectral analysis and a low specificity of evaluating the lesions’ stage [2, 4]. Therefore, signals in the red and near-infrared spectral ranges are being investigated as possible diagnostic indicators, where pigments such as porphyrins and melanin have fluorescent emission far from the bulk of other endogenous fluorophores [5].

On the other hand, researchers are looking for novel, fast, reliable and non-invasive techniques for tumor detection and evaluation of their type and stage of growth. Among the most severe cutaneous neoplasia is pigmented malignant melanoma (MM). It develops from melanocyte cells that produce the pigment melanin. Compared to other types of skin tumors, it is less common (about 10% of new cases annually), but it spreads extremely rapidly throughout the body (metastasizes) and therefore melanoma is one of the leading causes of death among patients worldwide. A huge problem is its difficult diagnosis at an early stage of its development, when the chances for effective therapy are relatively high. Its diagnosis at an early stage of tumor development is of low diagnostic accuracy and even experienced dermatologists define this disease with a 75-80% diagnostic accuracy due to its resemblance to other melanin-pigmented benign and dysplastic skin neoplasms. Due to the high metastatic activity of malignant melanoma, histological sampling carries the risk of spreading tumor cells through the bloodstream and spreading metastases throughout the body [6]. Therefore, new, objective, non-invasive diagnostic methods are sought, allowing high-precision diagnosis and differentiation of malignant melanoma from other forms of skin cancer or benign skin changes. Melanin, the pigment in this type of pathologies (MM), is a molecule strongly absorbing in the ultraviolet and visible spectral range, being a natural filter in our skin against the harmful short wavelength radiation of the Sun. However, in the near-infrared spectral region melanin has fluorescent properties and could be detected using NIR excitation [5, 7-9].

In this study, we investigated pigmented skin neoplasia – benign, dysplastic and malignant ones – using the NIR fluorescence technique in a steady-state mode and the fading of their fluorescence signals observed during the photobleaching process. Both the spectral and temporal parameters could be used to differentiate malignant melanoma from other types of pigmented cutaneous lesions [10].

2. Methods and materials
Fluorescent spectra from ex vivo pigmented cutaneous lesions were detected using single excitation at 785 nm of pairs of benign or malignant tissues and healthy skin from the safety area of samples surgically excised from 15 patients (benign (BN-5) and dysplastic (DN-3) nevi, as well malignant melanoma (MM-7)). The procedure of obtaining the samples included excision during surgery for removal of cutaneous neoplasia lesions. After the surgical removal, the lesions were divided in two parts – for histological and for spectral analyses. In the case of spectral measurements, the biological samples were transported under isothermal conditions and in a safe-keeping solution from the hospital to the spectral laboratory, where their fluorescence properties were investigated. A written informed consent was signed by all patients; the experiments were approved by the Ethics Committee of Tsaritsa Yoanna-ISUL University Hospital, Sofia.

The ex vivo examination of the skin lesions was performed by a laboratorial spectroscopic system for analysis of autofluorescent signals in the near infrared region. The experimental setup includes a diode laser (785 nm, 100 mW, CW), a USB 4000 portable spectrometer (Ocean Optics, Inc., Dunedin, USA) to which a fiber optic probe is connected for detection of the emission signal, and a computer (figure 1). The resolution of the spectrometer is 3.5 nm. The signal integration time for each spectrum is selected to be five seconds. The laser radiation is filtered from the detection channel of the fiber optic probe by a long-pass filter at 825 nm (OD4-825, Edmund Optics Inc.). The operating range of detection of the micro-spectrometer is 350 nm to 1050 nm.

For each sample, ten spectra of normal tissue, neoplasia, and the signal coming from the environment (light noise) were taken. Then we averaged the spectra obtained from different areas of the skin and
extracted the averaged noise signal. We thus obtained the final autofluorescence spectrum of the corresponding sample for further analysis.

![Figure 1](image-url)  
**Figure 1.** Photograph of the experimental set-up used for *ex vivo* NIR autofluorescence measurements of skin samples.

To record the presence and dynamics of photobleaching, we used the same experimental setup, but irradiated the samples for 10 minutes and recorded the corresponding fluorescence spectra every 20 seconds. Measurements were made at five points of tumor and healthy tissue and averaged for the respective tissue type.

### 3. Results and discussion

The interaction of light with the skin is a complex process with combined effects from the repeated scattering and absorption acts. The reason for this complexity is the multicomponent nature of the skin and its various layers. The presence of pathological and morphological changes in the skin caused by the development of a disease leads to a change in its fluorescence spectrum compared to that of normal skin tissue. When excited by UV-VIS light, the spectral range of the pigment melanin has a low quantum yield and its emission is very weak. Therefore, in this type of research, the endogenous fluorophores that contribute to the autofluorescence spectrum are various amino acids, proteins, coenzymes and others. On the other hand, when irradiated by a source in the near IR spectral range, the pigment melanin is the main fluorophore causing an autofluorescent signal. Various lipids (incl. lipofuscin) and endogenously contained porphyrins also contribute to the overall appearance of the autofluorescent signal in the near IR region [11].

![Figure 2](image-url)  
**Figure 2.** (a) Comparison of fluorescence spectra of different level melanin-pigmented normal skin, benign and dysplastic nevi, and pigmented malignant melanoma lesions and (b) basal cell carcinoma and normal skin comparison after excitation at 785 nm.

Figure 2 (a) compares the fluorescence spectra of normal skin and benign and malignant melanin-pigmented lesions. The fluorescence signal increases in dependence of the lesion severity, being the highest in the case of melanoma lesions. The autofluorescence spectrum from a non-melanoma tumor – basal cell carcinoma (BCC), is presented in figure 2 (b) in comparison with the spectrum of the surrounding healthy tissue. In both graphs 2a and 2b, a significant difference is observed in the intensity of the received signal. It can also be seen that the maximum emission of diseased and healthy skin tissue in both samples is at about 822 nm. We hypothesize that the increased fluorescence signal intensity in
the case of unpigmented basal cell carcinomas is due to the contribution of the emission of endogenous porphyrins that accumulate in this type of neoplasm.

Figure 3 (a) presents the absorption spectrum of melanin in melanosomes calculated according to the works of SL Jacques et al. [12, 13]. The absorption of the melanin in skin melanosomes is approximated by the formula $\mu_a = 1.70 \times 10^{12} \lambda^{-3.48}$ [cm$^{-1}$], where wavelength $\lambda$ is in nanometers [13].

![Figure 3](image)

**Figure 3.** (a) Absorption spectrum of melanin in melanosomes (b) Comparison of photobleaching dynamics of normal skin, dysplastic nevus and malignant melanoma lesions and (c) autofluorescence signal in the initial and the final moment of photobleaching process for dysplastic nevus and malignant melanoma using CW excitation at 785 nm.

The other experiment reported is related to registering the photobleaching process and studying its dynamics. Figure 3 (b) presents the decrease of the autofluorescence intensity of normal skin (phototype III), a dysplastic nevus and malignant melanoma, and figure 3 (c) presents the fluorescence intensities distribution of the emission maxima in the initial and final moments of photobleaching for all DN and MM lesions investigated. As mentioned above, the autofluorescence arising upon excitation in the NIR spectral range is caused by porphyrins, lipids, and most of all, by the pigment melanin. Since the respective lesions are highly saturated with melanin and normal skin does not contain such an amount of melanin, the observed difference in the dynamics of photobleaching corresponds mainly to the changes occurring in this pigment during the prolonged light irradiation. It has been observed that dysplastic nevi and melanoma maintain a very similar photobleaching tendency, similar to the pure melanin compound [14]. As a result, we cannot distinguish between the two diseases based solely on the data from the final moment of photobleaching. The diagnostically significant feature that helps in differentiating pigmented skin lesions is that at the initial moment they differ in the level of the fluorescent signal, namely, the initial intensity of the autofluorescent signal of the nevus is lower than that of malignant melanoma.

4. Conclusions

The diagnosis of early-stage malignant melanoma continues to be a serious clinical problem. The fluorescence-based techniques are perhaps among the most promising diagnostic tools. This is evidenced by the huge number of different studies in the field of fluorescence spectroscopy that have been conducted over the past two decades to show that this technique is a reliable diagnostic method that allows to distinguish between different types of skin lesions even at an early stage of their development. Unfortunately, in highly pigmented neoplasms, the standard approach that uses excitation sources in the near ultraviolet and visible ranges produces extremely weak fluorescent signals. Therefore, obtaining a significant signal requires excitation in another spectral range.

The experimental studies and the results obtained show that melanin in the skin exhibits noticeable autofluorescence signal upon excitation in the near infrared region. This pigment is crucial for the most severe form of skin cancer – malignant melanoma, which in turn shows that this type of research is promising and applicable for diagnostic purposes in the presence of pigmented skin lesions.
Autofluorescent photobleaching has potential for rapid and easy diagnosis and differentiation of dysplastic nevus from malignant melanoma, which is also of great clinical importance – for discrimination of the malignant from the transitional form of melanocytes. In combination with the technique of autofluorescence spectroscopy in the near infrared region, higher accuracy can be achieved. The combination of steady-state and photobleaching dynamics of fluorescence could be implemented in the clinical practice and it could be used as an additional tool for diagnosis of skin neoplasia.

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