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Autofluorescence and diffuse reflectance spectroscopy of pigment disorders in human skin

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Many up-to-date techniques based on recent progress in optics have been developed for medical applications. Fluorescence, absorption, and reflectance spectroscopy have been widely used as probes to acquire fundamental knowledge about physical, chemical, and biological processes. In the recent years, there has been growing interest in the common use of laser-induced autofluorescence (LIAF) and reflectance spectroscopy (RS) to differentiate disease from normal surrounding tissue – so called optical biopsy method. The goals of this work were investigation of cutaneous benign and malignant lesions by the methods of LIAFS and RS. A nitrogen laser at 337 nm was applied for the needs of autofluorescence excitation. Broad-spectrum halogen lamp (from 400 to 900 nm) was applied for diffuse reflectance measurements. An associated microspectrometer detected *in vivo* the fluorescence and reflectance signals from human skin. The main spectral features of benign lesions – compound nevus, dysplastic nevi and malignant lesions – pigmented, amelanotic and secondary malignant melanoma are discussed and their possible origins are indicated. Spectra from healthy skin areas near to the lesion were detected to be used posteriori to reveal changes between healthy and lesion skin spectra. Influence of the main skin pigments on the spectra detected is discussed and evaluation of possibilities for differentiation between malignant and benign lesions is made based on their spectral properties. This research shows that non-invasive and high-sensitive *in vivo* detection by means of appropriate light sources and detectors should be possible, related to real-time determination of existing pathological conditions.

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1. Introduction

Optical biopsy is relatively new term used in medical practice for description of fluorescence and/or reflectance spectroscopy of human tissues in vivo. Painless, instant diagnoses from optical biopsies will soon be a reality. These forms of optical diagnoses are preferable to the removal of several square millimeters of tissue surface - common in traditional biopsies – followed by delays while samples are sent for clinical analysis. Also for the early diagnosis of lesions (abnormal tissue) before they are visible to the eye, there can be a total optical examination of the area, instead of random, hit and miss sample selections. On top of this, optical biopsy apparatus only requires a learning curve of several practice attempts, compared to years of training needed for more conventional techniques [1, 2].

Autofluorescence spectroscopic technique is applied for early detection and/or differentiation of many tissue pathologies – atherosclerotic plaques [3, 4], aging, epidermal proliferation are detected by the LIAFS method [5-7]. Autofluorescence spectroscopy is one of these noninvasive methods that can identify diseases and increases the knowledge in medical diagnosis without using of additional contrast agents. However, fluorescence technique with or without exogenous fluorescent markers added find many applications - for monitoring of pathogenic bacteria photoinactivation processes [8], for the needs of dosimetry of photodynamic therapy procedures [4, 9, 10], for drug uptake analysis [10-12]. Fluorescent technique is also widely applied for cutaneous lesions' investigations, including erythema [5], psoriasis, vitiligo [13], and skin cancer [1, 4, 6]. This method gives information about biochemical composition of the tissue under study.

Diffuse reflectance spectroscopy from other side is responsible mainly about morphological information, which could be received from the tissues. Scattering intensity and spectral distribution of the signals detected could give information about scatterers' size and distribution (cells, nuclei, etc.). As the detected diffuse reflectance signal is superposition from diffuse scattering and absorption from tissues' pigments, the resultant spectrum also reveal information about main absorbers in the biological tissues, like hemoglobin and melanin in the skin and its pathologies [2, 14]. To benefit fully from reflectance spectroscopy's advantages, one needs to relate the spectral features with the morphology and biochemical composition of the tissue investigated. Data on a variety of human tissues, both in vivo and in vitro, have been published. Diffuse reflectance and backward scattering spectroscopy have been applied to characterize mineral loss in the teeth [15], characterization of ovarian tissue [16], evaluation of skin color and erythema doses [17], for skin cancer diagnosis [18, 19]. The results confirm that tissue reflectance spectroscopy provides valuable information on the tissue conditions.

In several systems for *in vivo* and *in vitro* studies both autofluorescence and diffuse reflectance spectroscopy have been applied. A combination of these types of

measurements has been employed for better understanding of the optical properties of normal and abnormal tissues and for increasing of sensitivity and specificity of lesions' diagnosis [2, 20, 21]. In the present paper, we also report the results obtained using combined fluorescence and reflectance spectroscopy methods, integrated as optical biopsy of the skin, applied to several different classes of benign and malignant non-melanin pigmented cutaneous lesions. In this study, autofluorescence and reflectance properties of common benign and malignant skin tumors are determined and origins of diagnostically significant spectral features are discussed. Differentiation scheme of benign/dysplastic/malignant pigmented skin lesions is also proposed.

2. Materials and methods

The system applied for spectroscopic measurements on human skin is described in details in previous work [2]. Optimum for excitation wavelength around 340 nm is found as most appropriate [1] for differentiation between autofluorescence signals from normal and neoplastic tissue, therefore a compact nitrogen laser emitting at 337 nm has been used as a most suitable excitation source. For reflectance measurements a halogen lamp with broad-band output spectrum (400-900 nm) was applied. Optical fibers were used to deliver the light from laser and lamp and to collect the fluorescence and reflectance signals. The spectra were recorded and stored using a fiber-optic microspectrometer (PC2000, Ocean Optics, Dunedin, FL, USA). A personal computer was used to control the system and to store and display the data using the specialized microspectrometer software OOI Base ("Ocean Optics", Inc., Dunedin, FL, USA).

For reflectance measurement mode initial calibration was carried out before every measurement. The portion of the light reflected from a sample is expressed as a percentage R [%] relative to a standard reflectance (such as a block of barium sulphate - $BaSO_4$). The reflectance R [%] is calculated by the following equation:

$$R_\lambda = \frac{S_\lambda - D_\lambda}{SR_\lambda - D_\lambda} \times 100\%,$$

where R_λ is the reflectance [%], S_λ the sample reflectance light intensity at each wavelength, D_λ the dark intensity at each wavelength (correction signal for detector dark current), SR_λ the standard reference reflectance intensity at each wavelength.

This routine calibration involves measuring an optical standard, in this case a block of $BaSO_4$. Such normalization eliminates the influence of the source and the detector response, both of which are wavelength dependent [22]. Immediately prior to each data collection session the fiber end was placed in front of a standard block to obtain the reference reflectance spectrum. Measurements of the dark current, D_λ, were performed by closing an external shutter in front of the spectrometer input. The dark and reference signals were then recorded automatically, after which the probe end was placed flat against the skin investigated.

Spectroscopic measurements of normal skin and lesion areas were carried out after 5-10 minutes of rest for each patient at room temperature (23 to 25˚C). Several spectra were measured from each suspicious area and averaged to reduce the influence of inhomogeinity of the lesions. It was recorded and averaged five to seven spectra from every lesion, depending on its size, and three to five spectra from surrounding normal skin and up to seven spectra from three different anatomical areas of human skin (palm, medial and lateral part of the forearm). These averaged spectra from the health skin area were used like an indicator of the spectral changes in the pathological areas. The spectra were smoothed using a Savitzky-Golay algorithm in order to reduce the instrumental noise of the spectrometric system. A constant distance between the end tip of the optical fibers and the skin surface were applied, using a mechanical stand to avoid any influence of displacement from the normal position on the intensity level. All spectra were obtained at normal incidence - at the 90^o angle between optical fibers end tip and skin surface. This geometry was carefully reproduced in all measurements to minimize fluorescence and reflectance intensity changes related to distance, and reflectance spectral shape changes related to the angle uncertainties during measurements [23].

Before every spectroscopic measurement a clinical observation and dermatoscopic evaluation of the lesion under interest was made. After these initial medical and spectroscopic measurements a histological samples were obtained from every lesion. The results from histological examination were used as a "gold standard" in comparison of all data obtained.

For the purposes of clinical diagnosis, the clinical variables were defined visually as follows: asymmetry, border irregularity, color and diameter of the lesion. For dermatoscopic diagnosis, all overall dermatoscopic impression was recorded for each of the lesions based on criteria in the recently published textbook [24]. The lesions were classified dermatoscopically (MoleMax II, DERMA Instruments). Each lesion was evaluated using ABCD scoring criteria as follows: Asymmetry (A), Border (B), Color (C) and Dermoscopic structures (D). The evaluation of differential dermoscopic structures was based on five main structural features: pigment network, dots, globules, and blotches (homogenous or structureless areas).

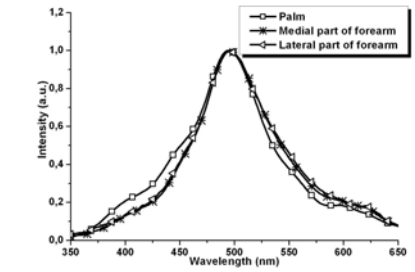
In this study we had included spectroscopic – fluorescence and reflectance results from 56 lesions, distributed as follow: 19 - benign nevi, 15 -dysplastic nevi, 12 - pigmented malignant melanoma (nodular and lentigo MM), 6 - amelanotic malignant melanoma and 4 - secondary melanomas.

3. Results and discussion

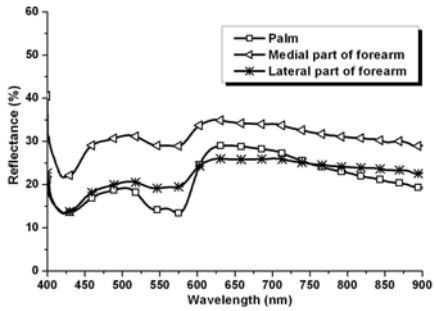
The data presented here are smoothed and averaged by lesion type fluorescence and reflectance spectra. Normal skin spectra, for comparison of the spectral changes occurring in presence of pathological conditions, are smoothed and averaged using all patients.

Every autofluorescence spectrum recorded *in vivo* is a superposition of fluorescence spectra of endogenous chromophores existing in the tissue distorted by photon re-absorption by the tissue pigments, mainly by blood and melanin. The spectral shape of normal skin fluorescence usually presents no significant differences from patient to patient. Intensity changes are more pronounced due to different skin phototype and anatomic area, as in both cases different level of melanin pigmentation could be observed. Detected slight differences in spectral shape are only for the case of palm skin fluorescence spectra versus other anatomic sites, where lack of melanin leads to deeper penetration of excitation and respectively for emission light. In this case influence of hemoglobin re-absorption of the fluorescence from deeper dermal layer is well pronounced. This effect is discussed in details in our previous work [25].

On the contrary, reflectance spectra of healthy skin from the different skin areas even from one patient showed significant differences in shape and intensity levels. Figure 1 illustrates the negligible influence on the shape of fluorescence spectra (fig.1a) and the strong influence on the reflectance spectral shape (fig.1b) depending on the measurement points' location for one patient. Fluorescence spectra are normalized with respect to maximum to reveal better possible spectral shape changes occurred.



a

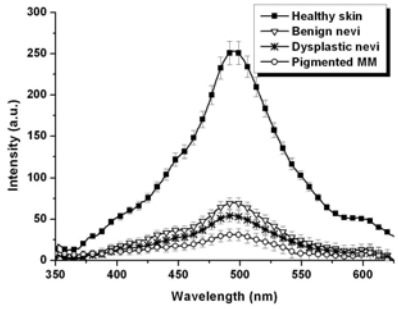


B

Fig. 1. (a) Fluorescence spectra of normal skin, normalized with respect to maximum, from different anatomic areas of one patient; (b) Reflectance spectra of normal skin from different anatomic areas of one patient, skin phototype I.

The medial part of the forearm is a region where the skin is not very tanned, so that the level of melanin produced as a reaction of the skin to the sunlight is low. The lateral part is the most tanned part of the human forearm because of the nearly permanent exposure to sunlight (increased level of melanin) and, therefore, has lower reflectance intensity than the medial part, where the level of melanin is much lower. The palm skin is the part of the hand richest in hemoglobin. In fact, the reflectance spectra minimums at 425, 540 and 575 nm, related to hemoglobin absorption are clearly observed in the palm reflectance spectrum. These minimums are also present in the forearm reflectance spectra, but they are overlapped by the melanin absorption from the epidermal layer. The decrease of the reflectance signal from the lateral forearm in the whole spectral region, as compared with the medial forearm is caused by melanin absorption.

Spectra from melanin-pigmented skin lesions, such as benign, dysplastic nevi and pigmented malignant melanoma revealed very low fluorescence intensities without significant spectral shape changes, as long as the reflectance spectra detected from this class of lesions present significant changes not only in the intensity level, but in the spectral shape. Thus, the reflectance spectra are more informative for discrimination between dysplastic and malignant forms of melanin-pigmented lesions, see Fig. 2.



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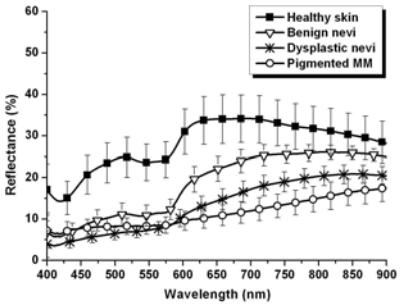
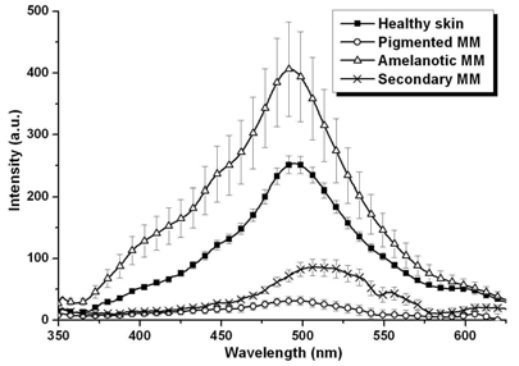


b

Fig. 2. (a) Fluorescence spectra of normal skin, compound nevus, dysplastic nevus and pigmented malignant melanoma (MM), averaged by lesion type for all patients; (b) Reflectance spectra of normal skin, compound nevus, dysplastic nevus and pigmented MM, averaged by lesion type for all patients; presented with their standard deviation.

In the Fig. 2a are presented averaged fluorescence spectra of normal skin, compared with melanin-pigmented

benign, dysplastic and malignant cutaneous lesions. Significant fluorescence intensity decrease, correlated with the type of pigment lesion, was observed for all lesions. The fluorescence intensities of the maximum of the two kinds of nevi investigated are very close one to another. The malignant melanoma fluorescence intensity is much lower than that of normal skin and nevi and could be separated from pigmented nevi spots. Using only intensity criteria for differentiation we obtain sensitivity higher than 80% for discrimination between nevi and MM lesions. However, no indicative discriminations of the fluorescence spectral shape between normal skin and nevi were observed. There are not indications about any significant accumulation of fluorescent chromophores in these types of lesions. Some slight spectral shape differences in malignant melanoma lesions are related to metabolic changes (NADH fluorescence increase in the region around 430-460 nm) and blood content increases (deeper minima at 420, 540, 575 nm).



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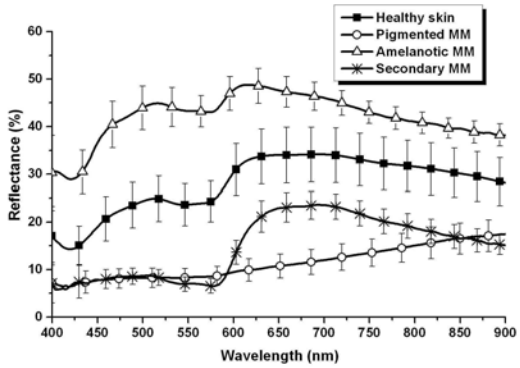

b

Fig.3. (a) Fluorescence spectra of normal skin, pigmented, amelanotic and secondary melanoma lesions, averaged by lesion type for all patients, presented with their standard deviations; (b) Reflectance spectra of normal skin, pigmented, amelanotic and secondary melanoma lesions, averaged by lesion type for all patients, presented with their standard deviations.

The mean values of the averaged reflectance spectra and their standard deviation for the different pigment skin lesions are presented in fig.2b. The benign compound nevus reflectance spectrum shows a significant decrease in the entire spectral region, best expressed in the blue region where melanin has stronger absorption than in the red region. Similar results are observed in the case of dysplastic nevus, but the intensity of the reflectance signal is lower. The malignant melanoma spectrum has the lowest total reflectance of all lesion types. Compound nevus and dysplastic nevus reflectance spectra are significantly different from those of melanoma. This difference in reflectance in blue and red spectral region could be even more pronounced if appropriate ratio between these spectral regions will be applied. In a previous work [23] we proposed such ratio used two wavelengths at 500 and 700 nm, related to specific combination of absorption properties for hemoglobin and melanin on these wavelengths. Using such ratio the sensitivity obtained for discrimination of the most interesting from clinical point of view pair - dysplastic nevus / malignant melanoma was found to be 82 % and specificity - 77 %.

Very interesting cases are these of amelanotic and secondary melanoma lesions, as there melanin pigmentation is not observed and the spectra detected are significantly different from pigmented MM or normal skin, see Fig. 3a and 3b.

From the best we know, for a first time in this work are presented autofluorescence and reflectance spectra of amelanotic malignant melanoma lesions and they are compared with the more common pigmented MM lesions. Amelanotic melanoma lesions are characterized by fluorescence and reflectance signals higher than that of the surrounding normal skin, due to lack of melanin in the tumour area. Visually amelanotic lesions are lighter than normal surrounding skin and using reflectance spectroscopy could be easily distinguished from it. Fluorescent signals could be also applied for such differentiation, as the standard deviations between these two kinds of spectra are not overloaded, and spectra could be distinguished, but the uncertainty of evaluation in this case is bigger. Recommended usage of reflectance spectroscopy for differentiation of amelanotic melanoma from normal skin is related to the significant rise of reflectance – higher than 35-40 % even in comparison with reflectance from skin phototype I normal samples as presented on figure 1b (for the medial part of the forearm) with the highest values about 30-34 %. From other side typical for normal skin is the highest reflected signal in the red spectral region, compared with the blue or green, which correspond to the color features of normal skin [26], as in the case of amelanotic lesions more pronounced blue component is observed. Using reflectance spectra of amelanotic lesions we received about 97 % diagnostic accuracy, only one spectrum of normal skin was misdiagnosed as a lesion.

Pigmented MM shows the lowest intensity related to the huge level of melanin content in these lesions, which

leads to a higher absorption of excitation and emission signals, as well as to lowest back-scattered diffuse light intensity. Secondary MM in the all cases observed during our clinical measurements visually has lower melanin pigmentation compared with pigmented primary melanoma lesions but higher level of hemoglobin content related to inflammatory processes and neovascularisation. The hemoglobin absorption is very well pronounced in both – fluorescence mode – as minima in the fluorescence signal at the region around 400-440 nm and narrower minima at 540 and 575 nm and in reflectance mode – as deep pronounced minima in the green spectral region 500-580 nm.

The skin tissue consists of many kinds of chromophores with different absorption and emission (if fluoresce) spectra, different quantum efficiencies, which have not been completely studied. The most common of them are melanin, haemoglobin and water. The water absorption spectrum covers ultraviolet and near- and far-infrared domains and it is not considered in this study. Melanin absorption has influence over the whole visible spectral region, which decrease slowly from blue to red spectral region. Therefore the fluorescence and reflectance intensity of the skin lesions decrease significantly with the increase of their melanin content [27] with higher distortion in the short wavelength region. This effect is clearly observed when one compares the fluorescence spectra of normal human skin with melanin-pigmented lesions. Other significant absorber is hemoglobin in the skin dermal layer. In visible spectral range it is characterized by strong absorption bands in the region of 420 nm, as well as in 540-575 nm region [6, 27]. Hemoglobin in its two forms, oxy-hemoglobin (HbO_2), and deoxy-hemoglobin (Hb), re-absorb the fluorescence of skin proteins and coenzymes in the autofluorescence signals. The hemoglobin influence is even more pronounced in the reflectance spectra as deep minima at 420 and 540-575 nm. This could be observed in the cases of neo-and hyper-vascularization in malignant lesions.

Chromophores, related to the formation of autofluorescence signals observed are mainly structural proteins, their cross-links, co-enzymes and lipids. The resultant spectrum detected in vivo is a superposition of these compounds with different effectiveness of manifestation for every one of them due to usage of only one excitation wavelength and differences in their quantum efficiency. Compounds, which fluoresce using 337 nm excitation, could be collagen type I – at 400-405 nm; its cross-links – at 460-490 nm; elastin – with maxima at 400-420, 460 nm; elastin cross-links – about 500 nm maximum of fluorescence; NADH – at 440-470 nm; keratin – at 430-460, and around 500-520 nm [4-6, 11, 12, 28].

Needs for precise knowledge of the normal tissue optical properties and their changes in different pathological conditions are closely related to the raising of the number of new cutaneous malignances worldwide. The skin cancer is one of the most spread tumors and despite of the results achieved recently in all clinical diagnostic techniques the most severe tumor - cutaneous melanoma continues to be an important problem of social health. For improvement of early diagnosis and for differentiation of risk lesions, an evaluation of spectral characteristics of several common cutaneous benign and malignant lesions is proposed. Pigmented melanoma may simulate benign lesions, including seborreic keratoses, heamangioma, compound and dysplastic nevi. Amelanotic malignant melanoma may clinically mimic a basal cell carcinoma [29]. Spectroscopic techniques applied in the study allow obtaining additional differentiation features between benign and malignant forms and could be used for development of diagnostic algorithms for lesion determination applicable in clinical practice. Our next step will be to cover other skin lesions using fluorescence and reflectance detection techniques and development of algorithms and equipment for early non-invasive skin cancer detection.

4. Conclusions

All clinical applications of optical biopsy in vivo are based on extracting information on the optical absorption, fluorescence and scattering properties of tissues by noninvasive measurement of the fluorescence diffusely-reflected light. These properties are related to the function or structure of the tissue. The fluorescence and reflectance spectroscopy of the human skin, combined as optical biopsy method, are very prominent for real-time determination of existing pathological conditions.

In this investigation we demonstrated the potential of optical biopsy for differential diagnosis of common benign and malignant cutaneous lesions. We also discussed some of the origins of spectral features in fluorescence and reflectance spectra obtained from investigated lesions. These results could be used for better comprehension of the skin optical properties and give wide range of possibilities related to early diagnosis and differentiation of cutaneous diseases.

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