

Optical Biopsy of Human Skin – A Tool for Cutaneous Tumours' Diagnosis

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Abstract: A short review of the recent status of optical biopsy of human skin as a tool for cutaneous tumour detection will be presented. Basically laser- and light- induced fluorescence and diffuse reflectance techniques incorporated in so called "optical biopsy" technique used for skin tissue analysis will be described and some original results and findings from our own investigations will be commented as well. Fluorescence spectroscopy gives information mainly about changes in the biochemical content, and diffuse reflectance about morphological changes and pigments' concentrations in the human skin. Both techniques are applied separately and as a common tool for early skin cancer diagnosis, give high accuracy and objective diagnosis, applicable into the clinical practice.

Skin optical biopsy diagnostic clinical trial is currently under implementation in the frames of University Hospital "Tsaritsa Yoanna – ISUL" and with broadening of the database with fluorescence and reflectance spectra of major skin benign and malignant pathologies we expect to receive objective tool for detection and evaluation of skin lesion type, which could become a basis for reliable system for skin cancer detection. Optical biopsy system is developed in the frames of Institute of Electronics, Bulgarian Academy of Sciences, and consists of autofluorescence and diffuse reflectance channels for early skin tumour detection. Fibro-optical probe allows receiving of noninvasive spectral information for the tissues' condition, which allows making highly-sensitive diagnosis of tissues in real time.

Keywords: Optical biopsy, Authofluorescence, Diffuse reflectance, Base-cell carcinoma, Malignant melanoma.

Introduction

Biomedical optics is one of the fastest growing areas of research. The non-ionizing nature of light applied for investigation and detection of abnormalities in human tissues make this area very attractive for development of new diagnostic techniques and modalities [1-2]. Many optical techniques are applied recently in clinical practice for obtaining qualitatively and quantitatively new data. Due to their high sensitivity in detection of small changes, spectroscopy techniques are widely used for detection of early changes in biological tissues.

Such techniques based on the recent progress in optics have been developed for laboratorial and clinical applications.

Optical biopsy is a summarizing term that describes various optical spectroscopic techniques applied for early diagnosis of tissue pathologies *in vivo*. Optical biopsy also could provide good diagnostic accuracy due to high spectral techniques sensitivity on small biochemical and morphological changes occurred in the tissues under interest [3-4]. The term “optical biopsy” could be found in the description of different optical spectral techniques, such as fluorescence, absorption, scattering, reflectance, Raman scattering, NIR luminescence of biological tissues, but its most popular mainly when light-induced autofluorescence and diffuse reflectance spectroscopic techniques for tissue detection and determination are described [2].

Light-induced autofluorescence spectroscopy of tissues

Light-induced autofluorescence spectroscopy (LIAFS) is a very attractive tool for early diagnosis of cancer due to its high sensitivity, easy-to-use methodology for measurements, lack of need for contrast agents’ application on the tissue under investigation, possibilities for real time measurements and noninvasive tumor detection [5-6]. It allows differentiation on the base of differences in biochemical content and metabolic state of the pathology. However, when the lesion is highly pigmented the obtained fluorescence signal is too weak to be used for diagnostics. In such cases exogenous fluorescent markers could be applied [7] or diffuse reflectance technique could be applied as well [8].

Nevertheless of all excellent features of the autofluorescence spectroscopy technique, till our days, no reliable and universal system for fluorescence detection of skin cancer has appeared on the medical market.

Problems for development of such diagnostic fluorescence system for skin cancer detection are related to the great variety of benign and malignant forms of skin pathologies, for example basal cell carcinoma (BCC) lesions have more than 15 sub-types, squamous cell carcinoma (SCC) lesions, have about 10 different subtypes, and all of them have variety of benign and dysplastic forms, as well as they are different, including by their fluorescence properties, on different stages on the lesion growth [6].

Good point here is the fact that one could use LIAFS for evaluation of the lesion stage, bad point is that he will need to compare this exact situation with great variety of other possibilities, such as lesion kind, stage of growth, and even patient skin general conditions, such as influence of medicines, ages, cutaneous phototype, etc. [9-10].

Broad variety of investigations is made to determine optical properties and sources of the autofluorescence signal coming from human skin tissues, which could be used for skin cancer diagnosis [5, 11-13]. Aromatic aminoacids – phenylalanine, tryptophan and tyrosine fluoresce, when deep ultraviolet (UV) excitation is applied (in the spectral region 260-300 nm). Structural proteins – collagen and elastin and their cross-links are responsible for the major part of skin autofluorescence, when excitation at 320-400 nm is applied. Using the same excitation region could be received the autofluorescence signals of co-enzymes, such as nicotinamide adenine dinucleotide (NADH) and flavins, which are indicators of metabolic alterations, that could be observed in the cutaneous tissue due to pathological changes. Other typical fluorophores in the human skin are also keratin, porphyrins, vitamins and lipids. In the Table 1 are presented major skin fluorophores and their excitation and emission maxima, according existing literature [5-6, 12-15].

Table 1. Major endogenous cutaneous fluorophores

Compound	Excitation max, nm	Emission max, nm
<i>Amino acids</i>		
Phenylalanine	260	280
Tyrosine	275	300
Tryptophan	280	350
<i>Structural proteins</i>		
Collagen	320-350	400-440
Elastin	290-325	340, 400
Collagen cross-links	380-420	440-500
Elastin cross-links	320-360, 400	480-520
Keratin	450-470	500-530
<i>Enzymes and co-enzymes</i>		
NADH	290, 350-370	440-460
NADPH	340	460
FAD, flavins	450	500-540
<i>Vitamins</i>		
Vitamin A	327	510
Vitamin D	390	480
Vitamin K	335	480
Vitamin B₆	320-340	400-425
<i>Lipids</i>		
Phospholipids	435	540, 560
Lipofuscin	340-390	430-460, 540
Ceroid	340-395	430-460, 540
<i>Porphyryns</i>		
Porphyryns	400-450, 630	635-690, 704

Autofluorescence technique is applied for diagnosis and differentiation of skin pathologies, such as vitiligo [16], psoriasis, benign, dysplastic and malignant lesions [5-6, 13, 17]. Different research groups report diverse results on the applicability of autofluorescence for cutaneous tumour detection and differentiation. Sterenborg and group [18] had examined the feasibility of autofluorescence spectroscopy for skin cancer detection using excitation at 375 nm. They did not observe significant differences in the shape of fluorescent spectra, or significant differences of fluorescence intensity values for tumor and normal skin. However, other groups reported more promising results, such as Brancaleon et al. [19], who observed higher fluorescence intensity in non-melanoma tumors vs. normal skin, using UV excitation for the tryptophan residues, which could be a result of epidermal thickening in tumor site. In contrast, the fluorescence intensity associated with collagen cross-links was lower in tumors, because of erosion and degradation of the connective tissue.

Other research group (Panjepour et al.) [20] had used fluorescence spectroscopy with 410 nm excitation for detection of non-melanoma tumors *in vivo*. This group found correlation between cancer detection diagnostic accuracy and skin phototype of the patient. With increasing of cutaneous pigmentation the diagnostic accuracy for tumor detection and differentiation from normal skin falls down from 93% – for phototype I, to 78% – for phototype III.

Na et al. [13] observed lower fluorescence signal in basal cell carcinoma tumors, in comparison with normal skin fluorescence, which is also observed by the group of Zeng et al. [21], and our own observations [6, 22-23].

When autofluorescence is detected *in vivo*, researchers need to take into account the influence of cutaneous absorbers, which distort the spectral shape of the autofluorescence signal coming from the tissue due to its re-absorption. Most typical skin absorbers, which influence fluorescence in the UV-visible spectral region are melanin, bilirubin and oxy and deoxy-hemoglobin. Melanin is typical skin pigment, with unique absorption properties, as its absorption decreases exponentially from UV to near – infrared spectral region, without specific absorption bands. Haemoglobin in its two forms – oxidised and deoxidised has maxima of absorption in the region around 400-420 nm, and two maxima for oxidised form – at 545 and 573 nm, and one broad maximum at 550-580 nm for deoxidised form respectively. Bilirubin, which is a breakdown product of red-blood cells, is typically observed in some liver pathologies, such as bilirubinemia in newborns and liver infections. This pigment adds yellow color to human skin and its absorption maximum lies in the blue spectral region – around 460 nm. It's not typically observed absorber in the skin of cancer patients.

Usually the absorption of hemoglobin and melanin is pronounced in fluorescence spectra detected *in vivo* as additional minima of the fluorescent signal, corresponding to their absorption maxima, at 420, 540-580 nm, or general decrease of the autofluorescence intensity in more melanin-pigmented skin areas in whole spectral region [6, 16-17, 24].

When highly pigmented skin pathologies are investigated the fluorescence signals coming from the tissues are too weak to be detected or signal-to-noise ratio is so low that do not allow proper spectral analysis of the received spectra. In this case diffuse reflectance spectroscopic technique is better to be applied.

Diffuse reflectance spectroscopy of tissues

Diffuse reflectance spectroscopy (DRS) 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, 25].

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 (by the reflective brightness of the tooth surface and other optical effects of mineral tissues, such as transmission and color absorption) [26-27]. This method is used for detection and characterization of ovarian tissue [28], cervical precancer [29] and adenomatous colon polyps [30]. In skin investigations reflectance spectroscopy is applied in evaluation of skin color and erythema doses [31], for skin cancer diagnosis [32-33]. The results confirm that tissue reflectance spectroscopy provides valuable information on the tissue condition.

Anisotropy, as well as phase measurements in the absorption and scattering spectra of different tissues are used for quantification of absorption and scattering coefficients induced

by biological pigments and structures, respectively [34-35]. Spatially resolved diffuse reflectance measurements are used for determination of the optical scattering and absorption coefficients of biological tissues [36-38], which describe fully the optical properties of the tissue in question.

Investigation of reflectance spectra obtained from microscopic tissue volumes can yield information about fine tissue structure, nuclear size distribution and other features of nuclei and other cellular organelles. Backscattered photons can be used to measure particle size. This will provide a useful tool for *in vivo* diagnosis of precancerous changes in the epithelium – enlarged nuclei are primary indicators of cancer, dysplasia, and cell regeneration in most human tissues [39-40].

On a macroscopic level reflectance spectroscopy is used for detection of soft-tissue stretch and rough [41], which is an important problem in plastic surgery. The changes in the diffuse reflectance properties of skin due to mechanical deformation can be used to assess the tension in wound and tissue expanders and help surgeons to treat wounds by minimizing the scar tissue.

Skin color measurements by optical reflectance spectroscopy are another important part of skin tissue properties investigations. Physicians have been using the skin color as an indicator for many pathological conditions for centuries. These measurements correlate color and minimal erythema dose [42], as well as reflectance spectra with melanin and hemoglobin concentrations in the skin [43-44].

Diffuse reflectance spectroscopy could give information not only for morphological information from the tissues. In many cases it is used to receive indirectly the pigments' content in the biological tissues investigated. Absorption of different skin pigments – bilirubin [45], blood [46], melanin [47], can yield valuable information about pathological conditions, such as high cutaneous bilirubin levels in new-born infants, which could cause permanent brain damage or about minimal erythema dose in skin (redness [46] or pigmentation [47] measurements). Absorption of these pigments cause significant distortions in the spectral shape of the diffuse reflected signals coming from the tissues and that changes have high diagnostic value.

DRS in skin cancer investigations is applied mainly for the melanin-pigmented cutaneous pathologies, including malignant melanoma (MM), as well as a part of the combination of two spectral techniques simultaneously with fluorescence, which allow increasing of the diagnostic accuracy in general for all pathologies investigated.

DRS is one of the optical techniques, with promising future for its application for the skin lesion investigations. Reflectance spectroscopy is utilized for investigation of pigmented skin lesions [48-49], making it possible to differentiate between melanoma and benign pigmented skin areas (e.g. moles, freckles, etc.). Many people have pigmented skin spots that may be confused with melanoma; differential diagnosis therefore is very important. To the experienced dermatologist the differentiation between melanoma and other lesion is straightforward, but it is increasingly difficult for melanomas in the early stages.

Diffuse reflectance signals could be applied for absolute determination of absorption and scattering coefficients of biological tissue and give reliable results for wide range of wavelengths. Reflectance spectroscopy of biological tissues has many advantages, related to

relative simplicity of the technique applied, possibilities for quantitative evaluation of pigments and other chromophores consisted in the tissue under interest, as well as possibility to be applied for real time non-invasive diagnostics method for determination of tissue pathology type.

Reflectance spectra obtained from a tissue under study have a specular and a diffuse component, see Fig. 1. The direct reflection by the skin surface is called specular (or regular) reflection, and is related only to the refractive index change between air and the epidermis layer. In practice, it also depends strongly on the exact surface and illumination conditions because of surface optical irregularities and roughness [50]. Another component consists of specular reflectance from deeper skin layers and a backward diffused scattering component. The scattering in tissues is due to discontinuities in the refractive index on a microscopic level, such as cell membrane, mitochondria, nuclei and other cell organelles, as well as collagen, elastin and keratin fibrils within the extra-cellular matrix. The reflectance spectrum obtained is corrected with respect to the incident light spectrum by taking into account the influence of different skin absorbers. The absorption in tissues in the visible and infrared spectral region is due to natural absorbers such as hemoglobin, bilirubin, melanin, and water. The nucleic acids, urocanic acid, proteins, melanin and their precursors absorb in the ultraviolet spectral region [49].

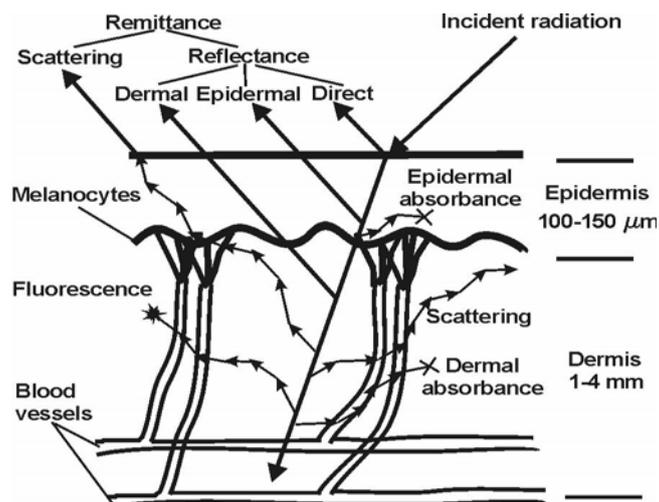


Fig. 1 Schematic cross section of human skin – description of light interaction with skin layers. Reflection remittance is the total radiation returned from within the skin tissue.

When one measures *in vivo* diffuse reflectance spectrum from the patient' skin, using optical fiber probe, he actually measures remittance part of the spectra. The influence of the skin optical probe geometry could be tremendous [51] and need to be taken into account when analyze the spectral results.

Optical biopsy – conception and applications

Optical spectra provide biochemical and morphological information about tissue under investigation, based on its absorption, elastic and Raman scattering properties [1-2, 17]. Fluorescence, absorption and diffuse scattering spectroscopy have been widely applied as probes to acquire information about physical, chemical, or physiological processes in the tissues. Optical diffuse tomography and optical coherent tomography are applied as effective imaging methods for revealing of structure of the tissues. These methods are proposed to be used by medical community for extension of possibilities of standard diagnostic modalities,

which are already introduced in the clinical practice, as x-ray, magnetic resonance and ultrasound imaging.

Optical biopsy is relatively new term used in medical practice for description of spectral techniques applied for early diagnosis of different tissue pathologies *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. In general, the predictive accuracy of optical biopsy is also better than prediction based on biopsy solely [3-4, 52]. On top of this, optical biopsy apparatus only requires a learning curve of several practice attempts, compared to years of training needed for some more conventional techniques. LIAFS and DRS are ones of the most perspective optical techniques, proposed to be introduced in medical diagnostic practice as main “optical biopsy” clinical tools.

Laser-induced autofluorescence spectroscopy (LIAFS) could be utilized to quantify differences between normal and abnormal tissues *in vivo*, providing appropriate method for detection of pathological lesions in real time. Diffuse reflectance spectroscopy (DRS) also allows distinguishing of pathological areas from normal tissue surroundings. These two techniques provide additive information to each other. LIAFS has revealed very high sensitivity for low-pigment pathologies diagnosis and differentiation by type, and it is almost useless for diagnosis of pigmented melanoma lesions, based only on its autofluorescence signal, due to low level of the fluorescence detected from such kind of lesions. DRS is irreplaceable for highly pigmented lesions, but reveal moderate sensitivity and specificity for unpigmented or low-pigmented cutaneous neoplasia. Therefore many investigators' groups that apply only one of these techniques could report controversial comments about their feasibility to be used as a clinical tool for early cutaneous lesions detection and differentiation. Their results strongly depend from the group of lesions investigated and technique applied. If they try to observe and diagnose pigmented pathologies and to differentiate malignant melanoma lesions from benign and dysplastic nevi, for example, using reflectance spectroscopy, such groups report excellent results (higher than 90% sensitivities and specificities) [48, 53]. In the same time, if pigmented basal cell carcinoma is detected only using fluorescence spectroscopy approach – the results of diagnostic analysis will be very moderate and will not reveal the diagnostic capacity of this detection technique, which leads to report about its low efficiency and poor clinical applicability [18].

Therefore, in the recent years, there has been growing interest in the common use of light-induced autofluorescence and reflectance spectroscopy to differentiate disease from normal surrounding tissue – mainly for detection and differentiation of cancerous and pre-cancerous changes in human body. 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 [5, 54-55].

Obtained sensitivity and specificity values could also strongly depend from the spectral features taken into account in differentiation algorithms applied from different researchers. Reflectance measurements applied for determination of different pigmented skin lesions use

specific features of the spectra obtained from normal skin, benign and malignant lesions, namely, mean value for specific wavelength(s), slope of the spectrum in one or more spectral ranges, integral value of the reflected signal for specific wavelength region, etc. Depending on the comparison between different special features used by different scientists, the sensitivity (SE) and specificity (SP) of the reflectance spectroscopy technique can vary in a wide range. Thus the values of SE: SP cited in different works are 76:87 [49], 80:46 [56], 83.6:90.8 [48], 89:88 [57], 90.3:77.4 [53] and 91:84 [48]. In our own investigations we observed this peculiarity and investigated the influence on the sensitivity and specificity diagnostic values depending from the spectral technique or their combination applied for diagnosis of given set of lesions [58]. Best results were achieved, when combination of laser-induced autofluorescence and diffuse reflectance spectroscopy are commonly applied for determination of malignant melanoma lesions. Diagnostic accuracy achieved was 90%, sensitivity and specificity values were 100% and 93.3% respectively, which was an excellent result in comparison with the diagnostic values, reached by other non-invasive clinically applied techniques (about 60%-70% for diagnostic accuracy).

Our investigations are a part of clinical trials for introduction of spectral diagnostic system for skin cancer detection in the common practice of the dermatological departments of Bulgarian hospitals. Skin “Optical biopsy” diagnostic clinical trial is currently under implementation and with broadening of the database with fluorescence and diffuse reflectance spectra of major skin benign and malignant pathologies, we expect to receive objective tool for detection and evaluation of skin lesion type, which could become a basis for reliable system for optical biopsy detection of cutaneous neoplasia. Here, we will present shortly some of our observations from the practice to present clinically the review of LIAFS and DRS techniques for skin cancer diagnostic applications.

Materials and methods

Investigations presented are a part of a clinical trial for introduction of spectroscopic diagnostic system for optical biopsy of skin cancer in the common clinical practice of dermatological department of University Hospital “Tsaritsa Yoanna – ISUL”.

Optical biopsy system

For LIAFS measurements multiple wavelength excitation of the endogenous fluorescence of benign and malignant cutaneous lesions is applied. Initially, lesions are classified visually by experienced dermatologist and dermatoscopically using ABCD scoring criteria. Second step is detection of lesion’ and surrounding normal skin autofluorescence using different excitation wavelengths, namely 365, 385, and 405 nm, received from several narrow-band light-emitting diodes. Optical fiber probe is used to deliver the light from LEDs and to collect the fluorescence signals from the skin surface. It consists of 7 fibers in circular geometry. Central fiber is used for autofluorescence signal detection and it is connected to microspectrometric system and surrounding six fibers are used for delivery of excitation light from the LEDs to the skin under investigation. The same fiber-optic probe is applied for reflectance measurements. In DRS mode of examination broad-band halogen lamp is applied as illumination source. In such a way we obtain cutaneous diffuse reflectance spectra for the 380-900 nm spectral region.

Routine calibration involves measuring an optical standard, e.g., a block of Spectralon® (OceanOptics Inc., Dunedin, USA). The subsequent reflectance measurements of the skin are normalized with respect to this standard measurement. Such normalization eliminates the influence of the source and the detector response, both of which are wavelength dependent.

Immediately prior to each patient data collection session, the fiber end is placed in front of a Spectrslon® block to obtain the reference reflectance spectrum. Measurements of the dark current, D_λ , are performed by closing an external shutter in front of the spectrometer input. The dark and reference signals are then recorded automatically, after which the probe end is placed flat against the skin investigated. Both kinds of LIAFS and DRS spectra are recorded and stored using a fiber-optic microspectrometer (USB4000, Ocean Optics, Inc., Dunedin, FL, USA). A personal computer is 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).

Several spectra are measured from each suspicious area and averaged to reduce the influence of inhomogeneity of the lesions. It is recorded and averaged five to seven spectra from every lesion, depending on its size, and three to five spectra from given area of normal skin. These averaged spectra from the health skin area are used like an indicator of the spectral changes in the pathological areas and for evaluation of influences, related to inter- and intra-patients spectral variations. The spectra are smoothed using a Savitzky-Golay algorithm in order to reduce the instrumental noise of the spectrometric system.

Clinical and dermatoscopic analysis

Before every spectroscopic measurement a clinical observation and dermatoscopic evaluation of the lesion under interest is made by experienced dermatologist (E.P. and/or P.T.). After these initial medical and spectroscopic measurements cytological and/or histological samples are obtained from every lesion. The results from histological examination are used as a “gold standard” in comparison of all data obtained. Up to now more than 400 patients are investigated with different skin pathologies, including 143 cases of basal cell carcinoma (BCC), 48 cases of squamous cell carcinoma (SCC), 12 cases of keratoacanthoma, 62 cases of malignant melanoma, dysplastic and benign lesions. Spectral properties of variety of benign cutaneous lesions are also evaluated for development of more precise discrimination algorithms for diagnosis of cancer lesions. All subjects were Caucasian volunteers – patients of the National Oncological Medical Center – Sofia and University Hospital “Tsaritsa Yoanna – ISUL”, with skin phototypes I, II and III according the classification of Fitzpatrick.

Results and discussion

Light-induced autofluorescence spectroscopy measurements of cutaneous tumors

Every autofluorescence spectrum recorded *in vivo* is a superposition of fluorescence spectra of endogenous fluorophores existing in the tissue [16-17, 52] 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 [6]. Autofluorescence spectra of human normal skin using different excitation sources, which emit on different wavelengths also reveal spectral shape changes, due to different effectiveness for excitation of given kind of tissue fluorophore, which could be excited in a given spectral range. This effect leads to set of autofluorescence spectra, typical for the normal skin tissues, which are discussed in the literature and observed in our own

investigations as well [59]. In comparison of fluorescence spectra using different excitation wavelengths is clearly observed appearance of new emission maxima and changes in fluorescence intensity, depending on absorption for given excitation wavelength of exact fluorophore. On the Fig. 2 are presented fluorescence spectra, normalized with respect to maximum for several excitation wavelengths. The normalization is applied to reveal better the spectral shape changes occurred.

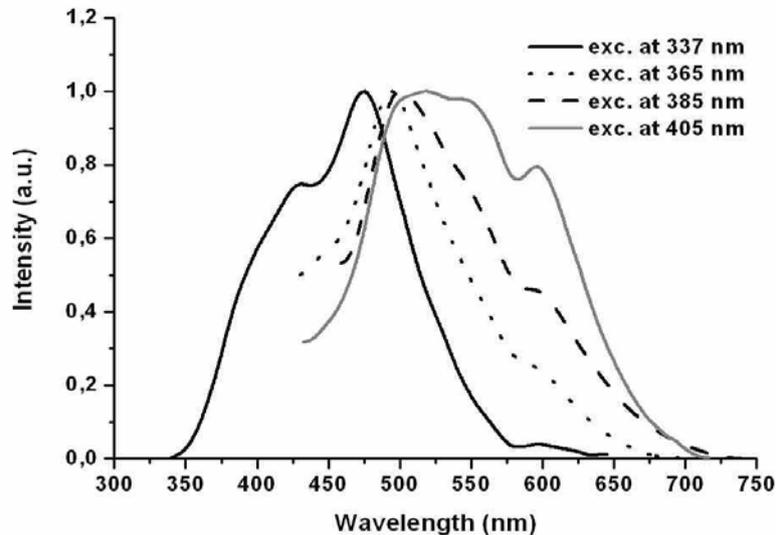


Fig. 2 Normalized with respect to maximum fluorescence spectra of normal human skin from one volunteer at different excitation wavelengths applied

With the increasing of the excitation wavelength, new fluorophores are involved in the shape formation of fluorescence spectrum. This is related to deeper penetration of long-wavelength excitation [1, 4], to higher absorption at given wavelength for some additional fluorophores and to differences in pigments influence, especially hemoglobin – the minima detected at 545 and 575 nm at Fig. 2.

However, light-induced autofluorescence spectra of normal skin for given excitation wavelength from different patients with the same phototype and from the same anatomic area detected are very similar [59] and shows very good repeatability by spectral shape and intensity features. The similar tendencies are observed as well when pathological changes occur, as spectral shapes and intensity trends are influenced in an analogous way for one given kind of pathology. For example basal cell carcinoma lesions always reveal fluorescence intensities lower than surrounding normal skin tissues, while squamous cell carcinoma usually has fluorescence brighter than surrounding skin. This observation could be used for differentiation of these two kinds of non-melanoma malignancies [21, 25, 54]. Malignant melanoma lesions have not any significant spectral shape changes, related to appearance of new fluorophores in their cells, but always have extremely weak fluorescence and by the level of the autofluorescence signal could be differentiated from dysplastic nevi with relatively good diagnostic accuracy – about 70%, which could be improved when reflectance spectral features of the lesions are also evaluated and could reach 90% [58].

When spectral shape changes are observed, they are related mainly to appearance or concentration changes in the fluorophores, which are presented in a given skin pathology, but could be as well an indicator of changes in metabolic activity of given tissue. Keratoacantoma lesions, which are pre-cursors of squamous cell carcinoma, are typical examples of fast

growing skin lesions, and there we have observed appearance of red fluorescence – in the region between 600 and 700 nm, related to endogenous porphyrins, which are accumulated in such metabolically active lesions, see Fig. 3.

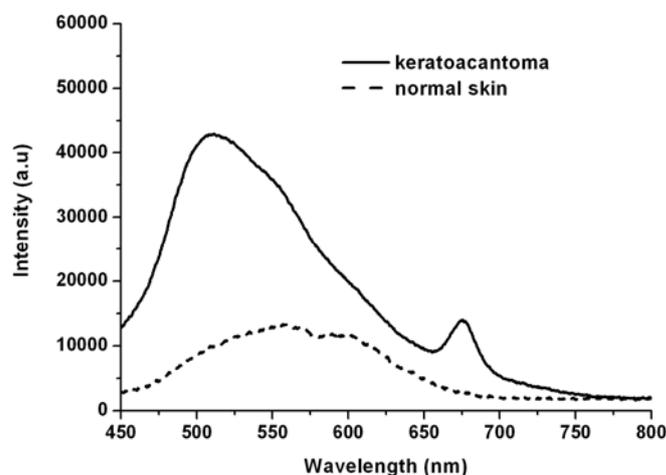


Fig. 3 Autofluorescence spectra of keratoacantoma and normal skin of a patient with cutaneous phototype III, using excitation at 405 nm

Endogenous porphyrins appearance is also typically pronounced in advanced stage of basal cell carcinoma lesions, as here the effect of accumulation is the opposite to the observed one in SCC growth – more advanced stage of BCC growth – higher fluorescence signal in the area near to 635 nm, related to endogenous porphyrins accumulation in the advanced stages of the pathology. There is well pronounced the spectral shape of the fluorescence, which is typical for protoporphyrin IX and other endogenous porphyrins – with maxima near to 630 and 700 nm [22]. This specificity of the autofluorescence signal for initial and advanced stage of the tumours is useful for treatment planning for patients with several lesions, which need to be treated consecutively, due to significant health problems and reduced immune response of the patient.

Another very important diagnostic feature, which is related to the autofluorescence spectroscopy measurements of the skin lesions, is its noninvasive character and possibility to scan large areas of the skin in real time, if necessary. Such clinical example is given on the next Fig. 4. Female patient, 67 years old with failed radio – and chemotherapy, with 3.8 cm x 7.6 cm size lesion on the forehead, diagnosed clinically and histologically as a BCC lesion, could not be cured for a period of about 2 years. After detailed scan of the lesion using LIAFS fiber-optical probe lesion was diagnosed as a mixed pathology with larger area of the tumour was a BCC and about 20% of the lesion – SCC area. After the discriminant spectral diagnosis obtained the therapeutic plan for this patient was changed in general, and tumor size was significantly reduced in the following six months according last information we have about this patient condition – lesion size is reduced to 0.6 cm x 1.5 cm.

Diffuse reflectance spectroscopy measurements of cutaneous tumors

Figure 5 shows reflectance spectra of healthy skin from the different skin areas of one patient. 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. Fig. 5 thus illustrates the strong influence of the measurement points location on the reflectance spectra measured.

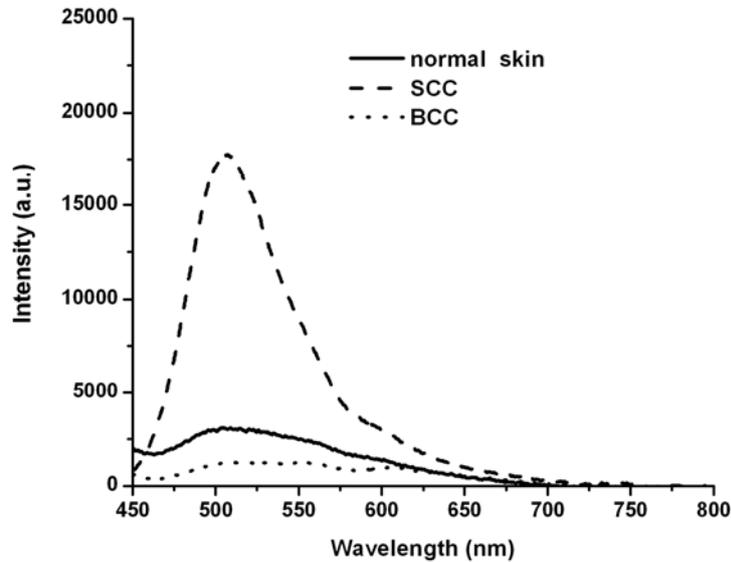


Fig. 4 Autofluorescence spectra of one patient with mixed tumour, including BCC area and SCC area of the lesion in comparison with the normal skin autofluorescence signal, excitation applied at 405 nm

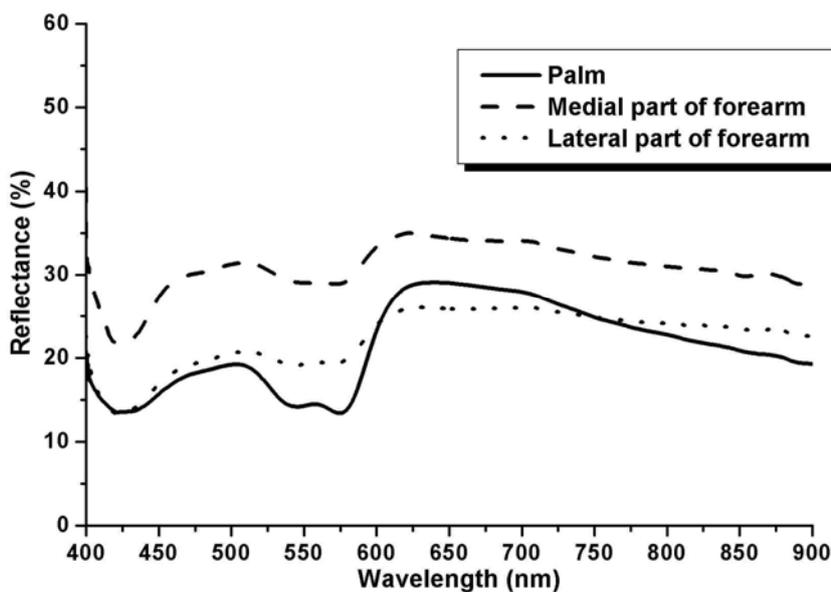


Fig. 5 Normal skin reflectance spectra from the medial and lateral parts of the forearm and the palm for one patient

The spectra obtained from normal skin in identical anatomic sites of different patients have similar spectral shape features, but differ in what concerns the reflectance intensity at different wavelengths, depending on the particular patient skin phototype. However, spectra obtained from normal skin sites near the pigmented lesion investigated change significantly from patient to patient as a result of the different spectral properties of the skin in the various anatomic sites (different melanin pigmentation, respectively absorption of the epidermal layer, or varied values of oxy- and deoxy-hemoglobin concentrations in dermal blood vessels). Fig. 6 shows averaged spectra for normal skin near the investigated lesion in ten different patients. These individual differences are related to the skin type, patient age and the particular measurement area on the skin surface and may affect the cutaneous lesion spectra, especially in the cases of not highly pigmented moles and spots. Thus, when developing a diagnostic algorithm, the normal skin features that are specific for each patient and each position of the lesion investigated must also be included.

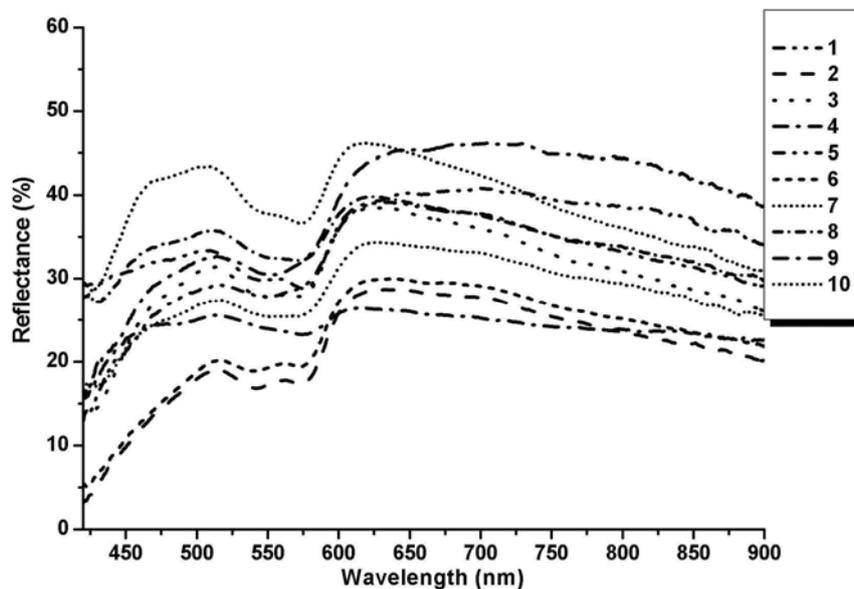


Fig. 6 Randomly chosen reflectance spectra of normal skin for ten different patients

The mean values of the averaged reflectance spectra and their standard deviation for the different pigment skin lesions are presented in Fig. 7. The benign compound naevus 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 naevus, but the intensity of the reflectance signal is lower. The malignant melanoma spectrum has the lowest total reflectance of all lesion types. Compound naevus and dysplastic naevus reflectance spectra are significantly different from those of melanoma.

One feature that the reflectance spectra of pigmented lesions show is a gradual decrease in the hemoglobin absorption peaks at 420, 540 and 575 nm in passing from normal skin to benign and dysplastic nevi to malignant melanoma, in which case the lowest values are observed. Another characteristic is the decrease of the reflectance spectra slope in the 420-500 nm region for all pigmented lesion types (benign, dysplastic nevi and malignant melanoma) as compared with normal skin. Also typical for the melanin-pigmented lesions is the change of the slope sign in the 650-900 nm spectral region from that of the normal skin reflectance spectra. These spectral features allowed us to differentiate easily all types of pigmented skin lesions from the normally pigmented surrounding skin, as well as to distinguish between

benign nevi and malignant melanoma, if we used the slope values in 420-500 nm spectral region; or to differentiate benign nevi from dysplastic nevi, when using the slope values in the 650-900 nm spectral region. However, the differences are not sufficiently significant to allow unambiguous simultaneous differentiation between all three groups of pigmented lesions investigated. From our previous investigations [60] more prominent spectral features for discrimination of these lesion groups are observed when compare the values at the 470-520 nm and 650-800 nm regions. The best results were achieved when the values of the ratio between normal and lesion reflectance intensity at 500 and 700 nm were used as a discriminating factor between different lesion types:

$$R = \frac{I_{norm}(500\text{ nm})I_{pigm}(700\text{ nm})}{I_{norm}(700\text{ nm})I_{pigm}(500\text{ nm})}$$

Sensitivity of 92% and specificity of 78% in discrimination between malignant melanoma from dysplastic nevi was achieved.

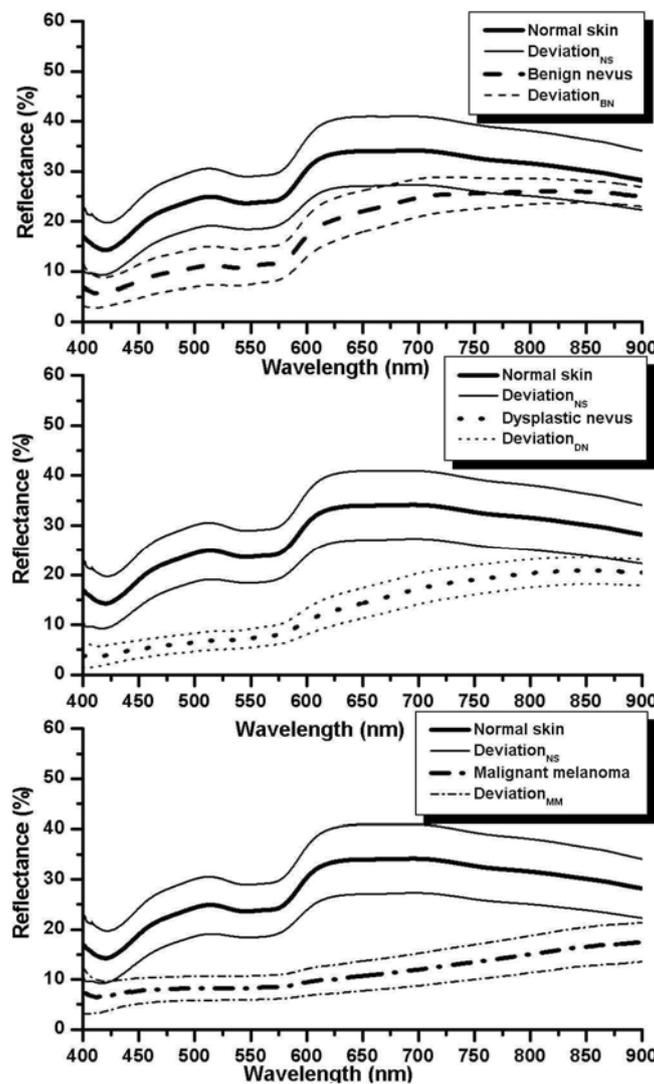


Fig. 7 Averaged reflectance spectra obtained from normal skin adjacent to lesions, benign compound naevus, dysplastic naevus and malignant melanoma with their standard deviation calculated for all lesions of one type

Conclusion

Melanoma incidence and mortality rates are on the increase in many countries. There is much evidence in the clinical practice that the standard biopsy could be reason for dissemination of the cancer cells and it is not advisable to be applied. In this context development of non-invasive, quick and reliable methods as optical biopsy attains significant importance. All clinical applications of optical biopsy *in vivo* are based on noninvasive extracting information on the optical absorption, fluorescence and scattering properties of tissues by highly sensitive measurements of the fluorescence and/or diffusely-reflected light. These spectral properties are related to its 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.

The most important for every diagnostic procedure developed is its possibility to differentiate malignant from non-malignant lesions. Where it is absolutely accurate, than 100% of the lesion types would be predicted. But every diagnostic test is imperfect in its own way – one procedure will miss many cases and make a few false diagnoses, another will miss a few cases, but the number of false diagnoses will be much higher. Using autofluorescence detection of skin benign and malignant pigmented lesions we obtain very good diagnostic performance for distinguishing of non-melanoma lesions *in vivo* from other simulating benign and malignant pathologies. Using diffuse reflectance spectroscopy we obtain excellent tool for pigmented pathologies differentiation, but it is a tool with moderate sensitivity for non-melanoma lesions detection. When these two detection techniques are applied in common and multispectral algorithms for diagnosis and differentiations are applied we rapidly increase the diagnostic accuracy of the received combined “optical biopsy” method. In all scientific reports observed it has been reported very good correlation between histological analysis of the skin and repeatability of the features of the fluorescence and reflectance signals from patient to patient with one-type lesion obtained from LIAFS and DRS measurements.

On the basis of the observed spectral changes, results of the current clinical trial, we could suggest that the used approach, related to creation of algorithms between specific wavelengths of obtained lesions and normal skin spectra can provide useful information on the given lesions that could be transformed into diagnostic algorithms for clinical usage. Clinical trial is currently under implementation and with broadening of the database with fluorescence and reflectance spectra of major skin benign and malignant pathologies we expect to receive objective tool for detection and evaluation of skin lesion type, based on its spectral properties.

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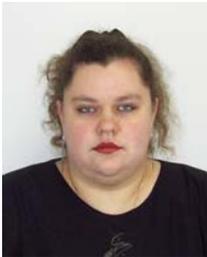
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