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COLLAGEN AS *IN VIVO* QUANTITATIVE FLUORESCENT BIOMARKERS OF ABNORMAL TISSUE CHANGES

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Collagen is an endogenous fluorophore that accounts for about 70% of all proteins of human skin, so it can be an optical marker for structural abnormalities in tissues registered by laser fluorescent diagnostics *in vivo*. Using the examples of such abnormalities as scars, scleroderma and basal cell carcinoma, this study shows the differences between coefficients of fluorescent contrast $k_f(\lambda)$ of abnormalities from the ones for healthy tissues at fluorescent excitation wavelength 360−380 nm. It is shown that scars and dysplasia are characterized by reduced values of $k_f(\lambda)$ for collagen. Due to high turbidity and phase heterogeneousness as well as variation of parameters of blood microcirculation and concentrations of other related chromophores, there is no mathematical model that precisely calculates the concentration of collagen in tissues only with the use of the value of fluorescent signal intensity. So, probably, the best marker of the pathological process is a comprehensive representation of $k_f(\lambda)$ for all endogenous fluorophores, i.e., for all used visible wavelengths. In this case identification of abnormal tissues is quite possible by detecting some deviations of coefficients $k_f(\lambda)$ for the optically identical and symmetrical regions of the human body.

Keywords: Medical diagnostics; spectroscopy; fluorescence; collagen; scars.

1. Introduction

The past 20 years have seen considerable advances in spectroscopic techniques allowing to register signals *in vivo* from a variety of endogenous fluorophores such as collagen, elastin, keratin, NAD·H/NADP·H, FAD and various porphyrins as well as lipofuscin (peroxidation product of intracellular organelles) which is accumulated during cell aging in human tissues.[1–3] *In vivo* fluorescent spectra acquisition method is simple, fast and relatively cheap. However, at present there is no commonly used technique to interpret spectral data definitively which limits the possibilities of spectral noninvasive photometric diagnostics in medical practice.

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Major obstacles in this respect are nonadditive signals of several fluorophores in one solution due to their mutual absorption and signal diffusion, high turbidity of biological tissues, their axial and radial heterogeneousness for the light beam and, therefore, nonmonotonic dependence of the resulting fluoroscence intensity from a fluorophore's concentration.[4,5] In a living organism, controlled variation of concentration of only one endogenous fluorophore is impossible without changing of concentrations of others,[6] which could be a way to draw up calibrating spectra directly. This fact presents just another obstacle for the researcher. At the same time one cannot help wondering how well-balanced a living organism is!

Therefore a great potential for noninvasive photometric research lies in vast clinical resources that present various distinct deviations in substance's concentrations in patients and allow researchers to analyze their influence on the resulting fluorescent spectra. One of the most common tissue's abnormalities is related to the collagen synthesis balance dysfunction. This abnormality manifests itself as various dysplasias, scleroderma as well as scar tissues. Scars are areas of fibrous tissue that replace normal skin (or other tissue) after injury. A scar results from the biological process of wound repair in the skin and other tissues of the body and constitutes a natural part of the healing process. Fibroblasts synthesize and secrete precursors of collagen and elastin which, outside cells, are transformed into collagenic and elastic fibers. Scarring is considered abnormal when the amount of fibrosis is excessive or suboptimal, as in cases of hypertrophic, atrophic or keloidal scars when it affects normal function and when it is symptomatic. The development of hypertrophic scars may be associated also with the wrong tactics of treatment. These scars are causing much concern and in need of correction as they distort the surrounding soft tissue. The fibers of the connective tissue of the dermis consist of the structural protein collagen, which is persistent biological material and ranges from 60% to 70% of the dry weight of tissue.

High concentrations of collagen are typical for sclerodermic skin.[7] However, it is not exactly the case with dysplasias. On one hand, in a case of the tumor cells divide and grow intensively and thus the share of extracellular collagen in these regions should be lower. On the other hand, if the tumor has a high dynamic of cell apoptosis, the regional collagen concentration can be higher. Enhanced concentration of all endogenous chromophores absorbing radiation can be observed in this case, and backscattering radiation may not be seen at all or can be seen only in part, complicating *in vivo* spectroscopic diagnostics.

In the present research, we narrowed down our region of interest to the study of collagen contribution to normal as well as abnormal tissue fluorescence. The primary objective of our study is to identify spectral differences in collagen fluorescence *in vivo* in normal skin and in skin with the above mentioned abnormalities (tumors and scars). This may help find out the future potential of the laser noninvasive fluorescence diagnostics in medicine in relation to diseases induced by collagen metabolism dysfunction.

2. Materials, Equipment and Methods

A LAKK-M multifunctional laser noninvasive diagnostic system was used in the present study. The system is equipped with four different lasers and an optical fiber probe with eight different optical fibers (four of them used for the laser radiation, the other four serve as receivers of backscattered secondary radiation). This system provides a complex noninvasive diagnostic technique: laser Doppler flowmetry, tissues reflectance oximetry as well as the laser fluorescent diagnostics.[8] Optical fiber's diameter is 2.5 mm. Distance between fibers inside the cord is around 0.6 mm. But depth of the tissue region to be diagnosed which can give an effective backscattered fluorescent signal depends on the laser excitation wavelength also. For instance, human skin penetration depth for 360−380 nm wavelengths (and linear size of the region to be diagnosed) is as low as 3−4 mm, for 532 nm wavelength — about 5 mm and approximately 1.5 cm for 632 nm laser.

However, according to various sources[1,2] maximum fluorescence of collagen in question can be obtained within the range of 380−440 nm at 340−380 ultraviolet excitation waveband. So, the probing region for collagen fluorescence equals to about 30 mm,[3] which is significantly lower than that for Doppler channel to study blood microcirculation parameters. Therefore the linear influence of the given factor on the fluorescence signal cannot be clearly identified in our case. The system LAKK-M is equipped with LED for excitation of fluorescence

at 370−380 nm and also with an optical filter for fluorescence studies which reduces a backscattered light at 360−380 nm by approximately 1000 times ($\beta \approx 1000$).

The so-called coefficient of fluorescent contrast $k_f(\lambda)$ is introduced to describe a fluorescence of various endogenous fluorophores quantitatively.[8] The analogous formula to calculate the coefficient $k_f(\lambda)$ is used in our present study:

$$k_f(\lambda) = \frac{I(\lambda)}{I(\lambda) + I_{\max\,\text{laser}}} = \frac{1}{1 + I_{\max\,\text{laser}}/I(\lambda)}, \quad (1)$$

where $I(\lambda)$ represents registered fluorescence intensity at wavelength λ, $I_{\max\,\text{laser}}$ represents the maximum intensity of the backscattered laser radiation (383 nm), registered and reduced by β times. One can see that the $k_f(\lambda)$ we use here is decreased by two compared to that in Ref. 8. It can be observed that $k_f(\lambda)$ in this case is within the range of 0 to 1. In a general case $I(\lambda)$ was used for each fluorophore at the corresponding wavelength λ, where maximum efficiency of its fluorescence would be expected.

Collagen (⟨Vita RINO⟩, cosmetic collagen) was used to register fluorescence properties of pure collagen. It is pure collagen-IV 97% dispersed in water. Essential tissue fluorophores in our study have the following characteristic points of fluorescent signal like it is shown in Table 1. Unfortunately, we cannot differentiate fluorescence signals of collagen and elastin *in vivo* and in practice their total contribution was observed at 422 nm wavelength under 360−380 nm excitation. However, it is well known from literature sources that the total molar and volume concentration of the collagen in a human skin is significantly higher than that of the elastin. Besides a collagen fluorescence spectrum has a wider right band, therefore, the signal at 442 nm wavelength can be identified as collagen contribution. In the same fashion due to signal interference distortions keratin and NAD·H signals overlap at 494 nm wavelength.

Table 1. Characteristic points of spectra and their interpretation in terms of endogenous fluorophores.

Wavelength, nm	Signal interpretation
383	Backscattered laser
422	Elastin and collagen
443	Collagen
466	Keratine/NADH
494	NADH
555	FAD
605	Lipofuscin
635	Porphyrins
704	Porphyrins

Over 30 patients with various forms of skin tumors in different regions of the body were tested during the preliminary research. Five patients with basal cell carcinoma (BCC) in different regions of the face, two patients with larynx BCC deep scar tissues were selected from them for the present detailed study. The selected patients were volunteers who at the moment of the study were being diagnosed and treated at MONIKI (Moscow Regional Clinical Research Institute) after M. F. Vladimirsky. Five relatively healthy people were involved in the study as well. For the purpose of the present study the sex, age as well as a localization of the tumors were not considered. Therefore the statistic analysis at the present stage of the research was not set as one of the objectives.

3. Results and Discussion

The first step of our study was to identify fluorescence characteristics of collagen-IV water solution. The following fluorescent spectrum was obtained (Fig. 1). It can be observed that apart from the device noise the registered spectrum is distorted by interference supposedly on the anti-blooming covering of the CCD of the spectrophotometer. However, this factor with a constant coefficient for each wavelength should not be considered as an obstacle when relative comparison of various spectra is being performed. Maximum fluorescence of pure collagen solution under the given circumstances is observed at 420−422 nm wavelength.

Fluorescence characteristics of normal skin of our five healthy volunteers are shown in Figs. 2(a) and 2(b). Considerable variation of the spectra in means of the signal amplitude and a correlation of various regions can be observed. Maximum of the skin fluorescence was always at 460−480 nm which to a larger extent corresponds to NAD·H and/or keratin fluorescence.

Fluorescent characteristics for five patients with the face skin BCC are shown in Figs. 3(a) and 3(b). As it is seen from the figures, the collagen fluorescent contrast coefficients for the given types

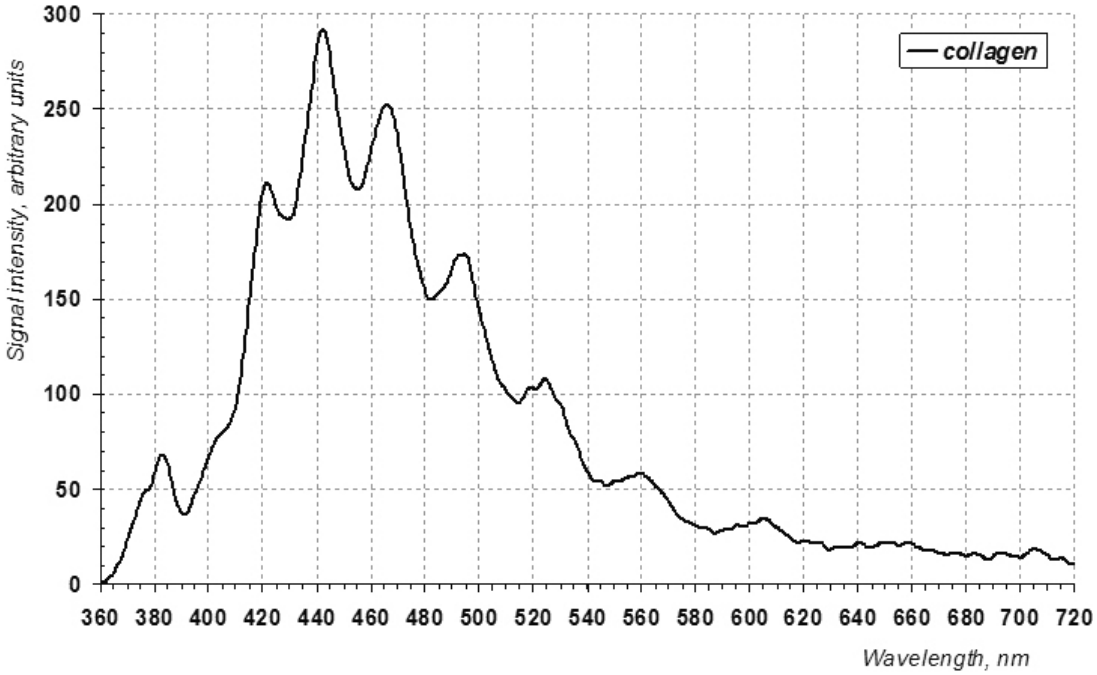
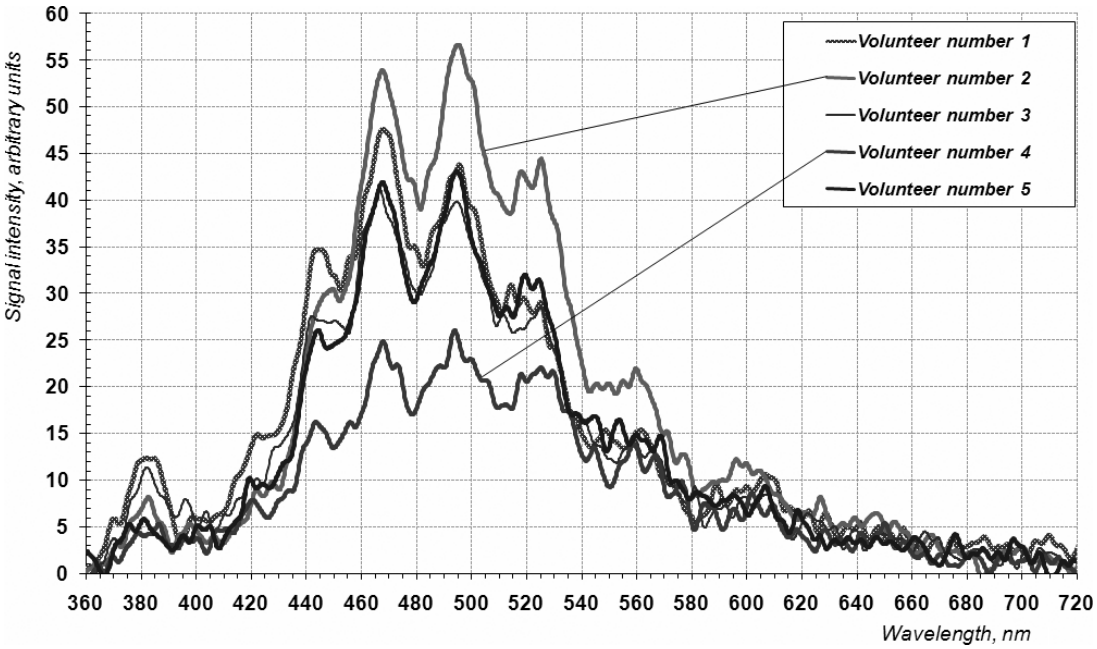


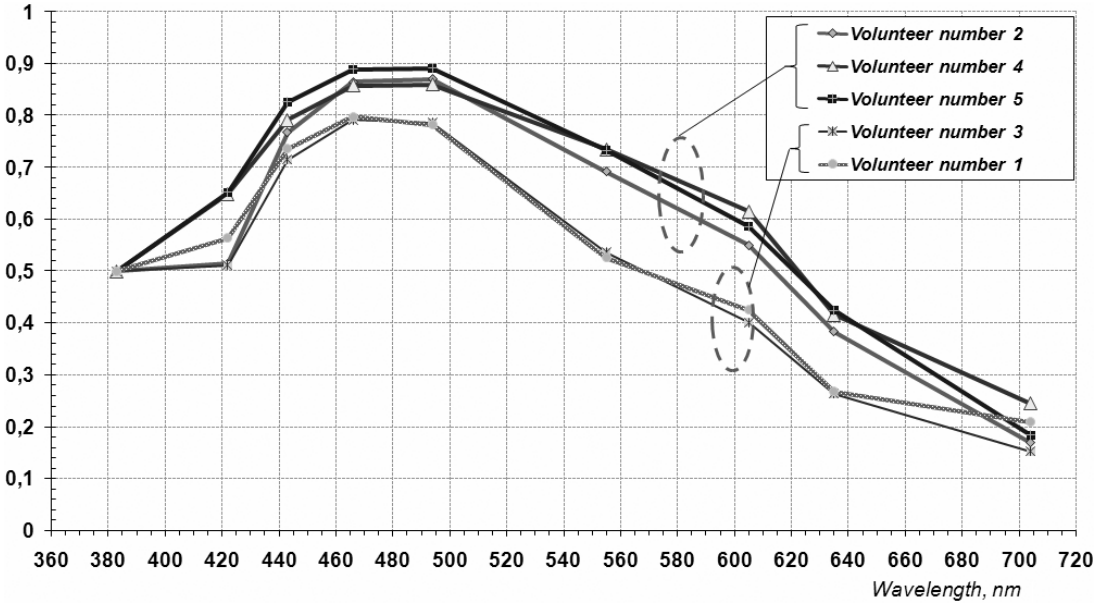
Fig. 1. Fluorescence spectra of collagen at 370−380 nm excitation.

of dysplasia can be clearly distinguished from those of normal skin. Absolute amplitude variability could have been caused by different tissue blood filling, different concentrations of other absorbing chromophores (melanin, bilirubin, etc.) as well as different tissue density and thickness of its structural layers in different regions of the body. Age specifics such as lower water concentration, higher



(a)

Fig. 2. (a) Fluorescence spectra of tissues in healthy volunteers. (b) Coefficients of fluorescent contrast of tissues in healthy volunteers for spectra in Fig. 2(a). No visible and significant differences.

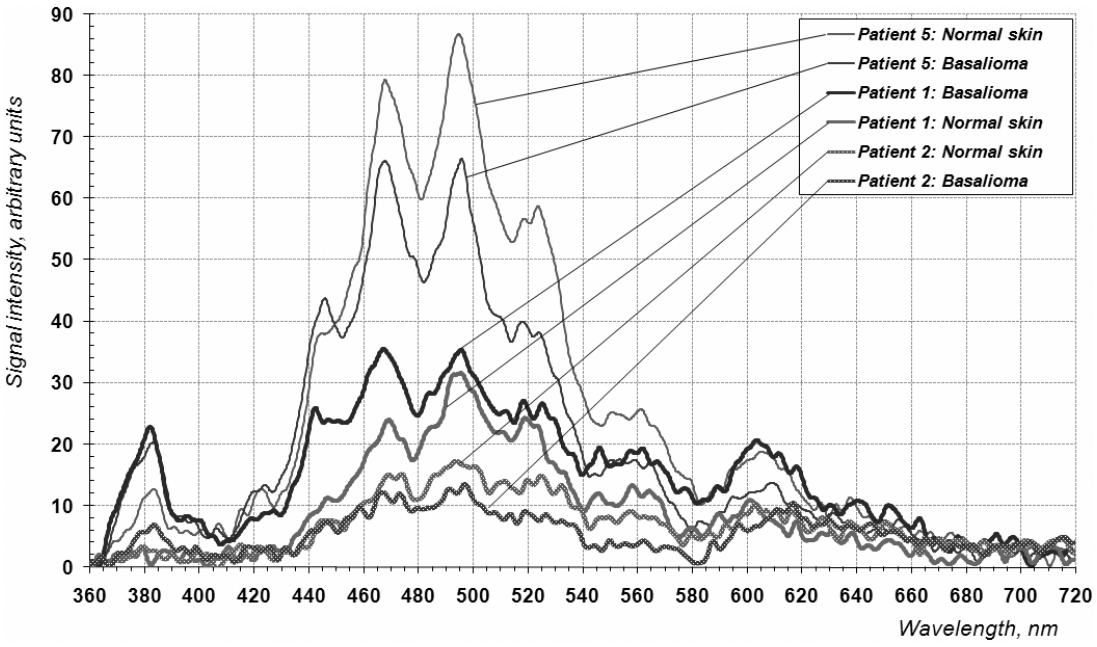


(b)

Fig. 2. (*Continued*)

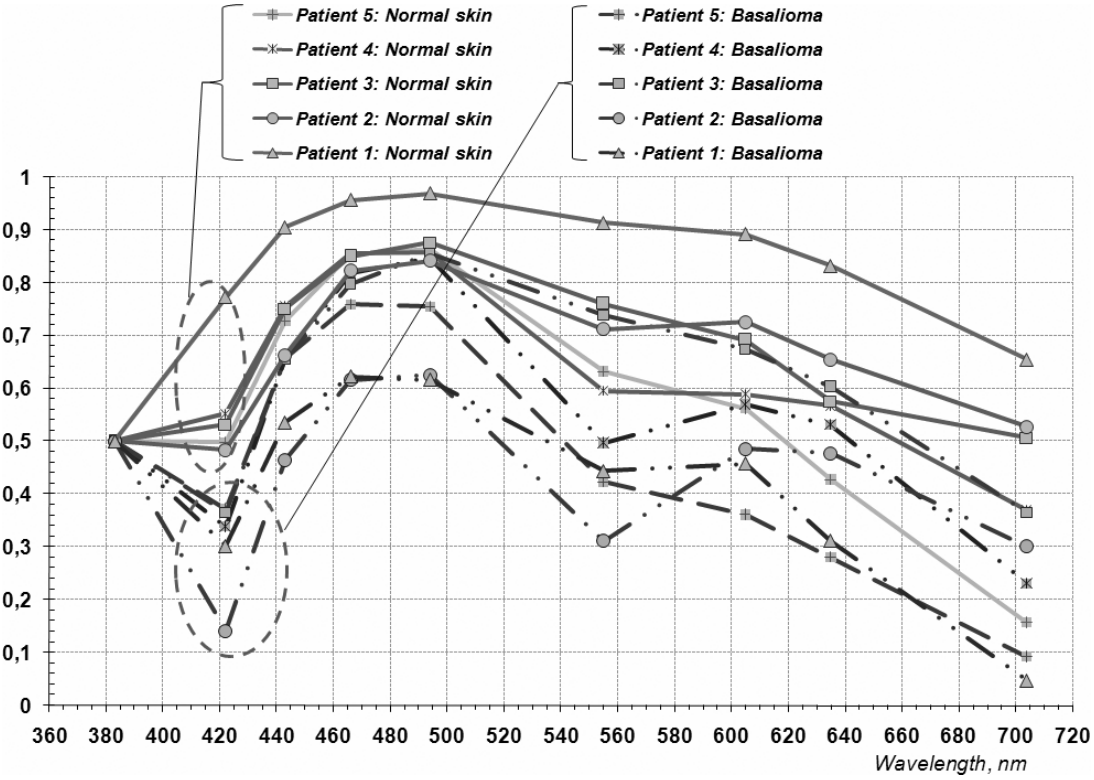
tissue turbidity, etc. can be taken into consideration. Therefore the criteria for "normal" fluorescent spectrum of a "normal" skin cannot be stated in our research.

As it was expected, in patients with scar tissue scars contribute considerably to collagen fluorescence compared with normal skin (see Fig. 4(a)), on the contrary, corresponding coefficients of the



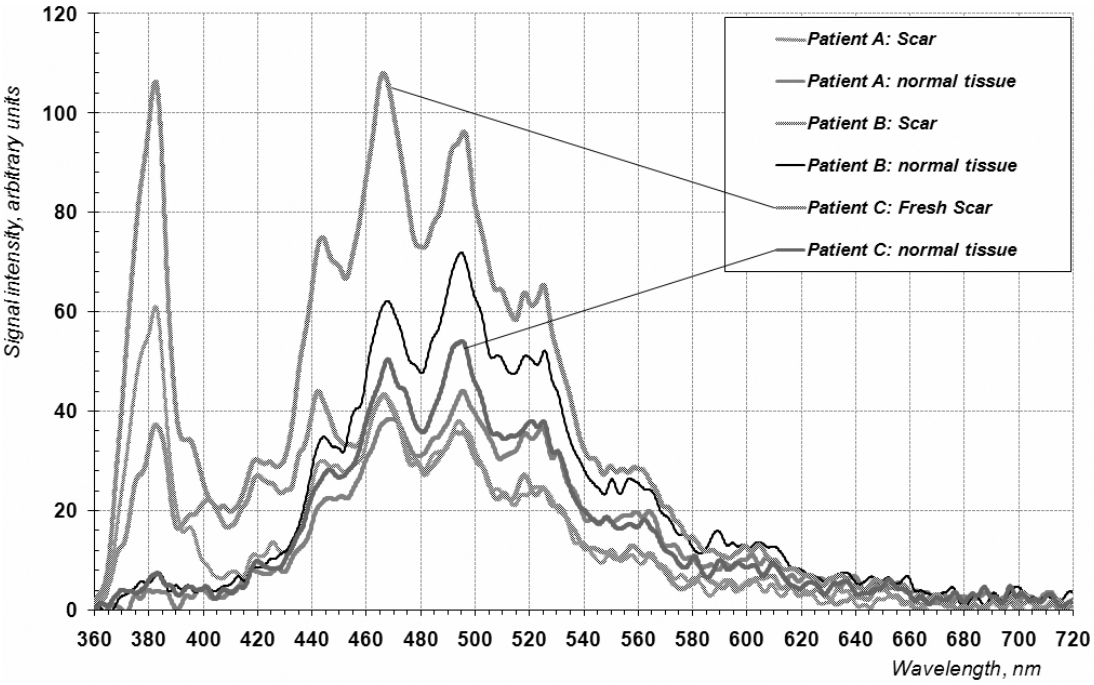
(a)

Fig. 3. (a) Fluorescence spectra of tissues in patients with BCC and from their symmetrical normal skin. (b) Coefficients of fluorescent contrast for different tissue in patients with BCC and for their symmetrical normal skin for the spectra in Fig. 3(a). Significant differences in the collagen fluorescence.



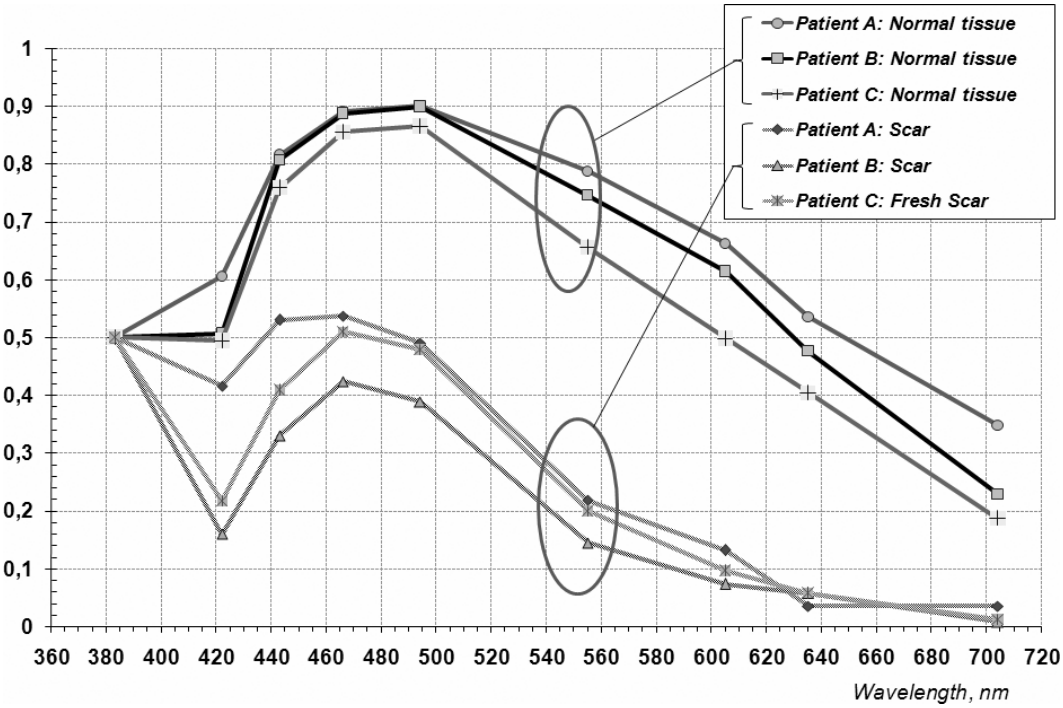
(b)

Fig. 3. (*Continued*)



(a)

Fig. 4. (a) Fluorescence spectra in patients with larynx stenoses (scars) and for their normal intact tissues. (b) Coefficients of fluorescent contrast for different tissues in patients with larynx stenoses and for their normal intact tissues for the spectra in Fig. 4(a). Significant differences in all spectra.



(b)

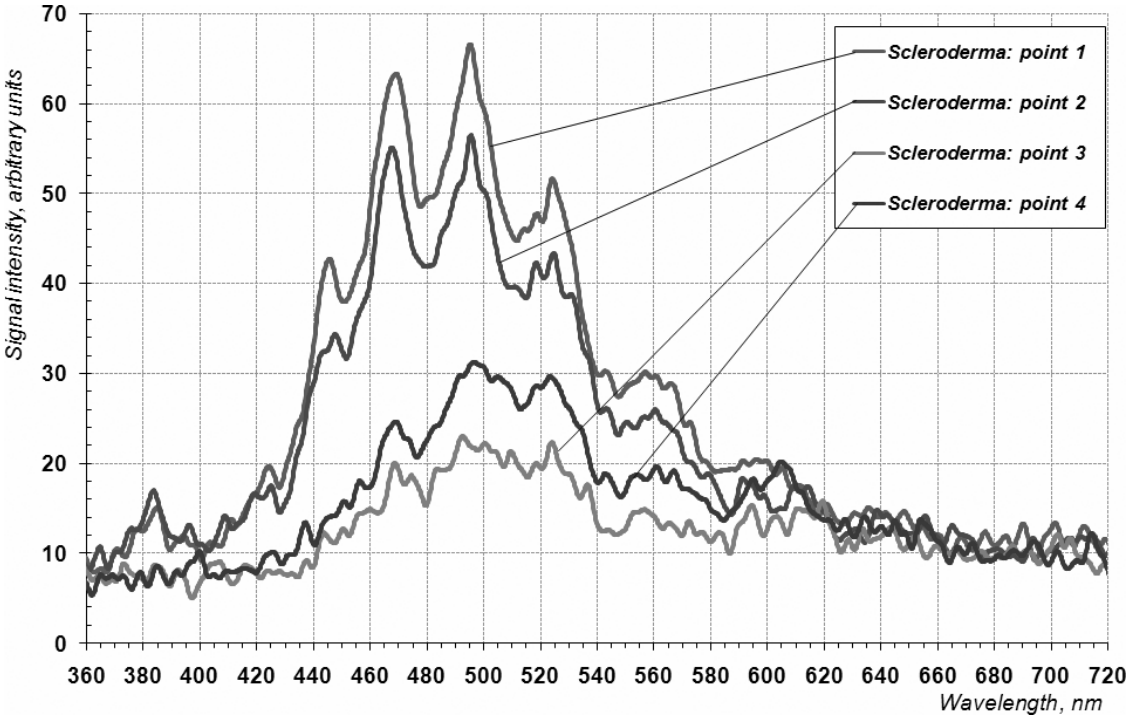
Fig. 4. (*Continued*)

fluorescent contrast are lower (Fig. 4(b)). This situation is similar to BCC tissues deviations and is caused by the increase of backscattered laser signal along with higher collagen fluorescence. This can be accounted for by relatively low concentrations of absorbing chromophores and probably by low dynamics of cells' apoptosis in tissue.

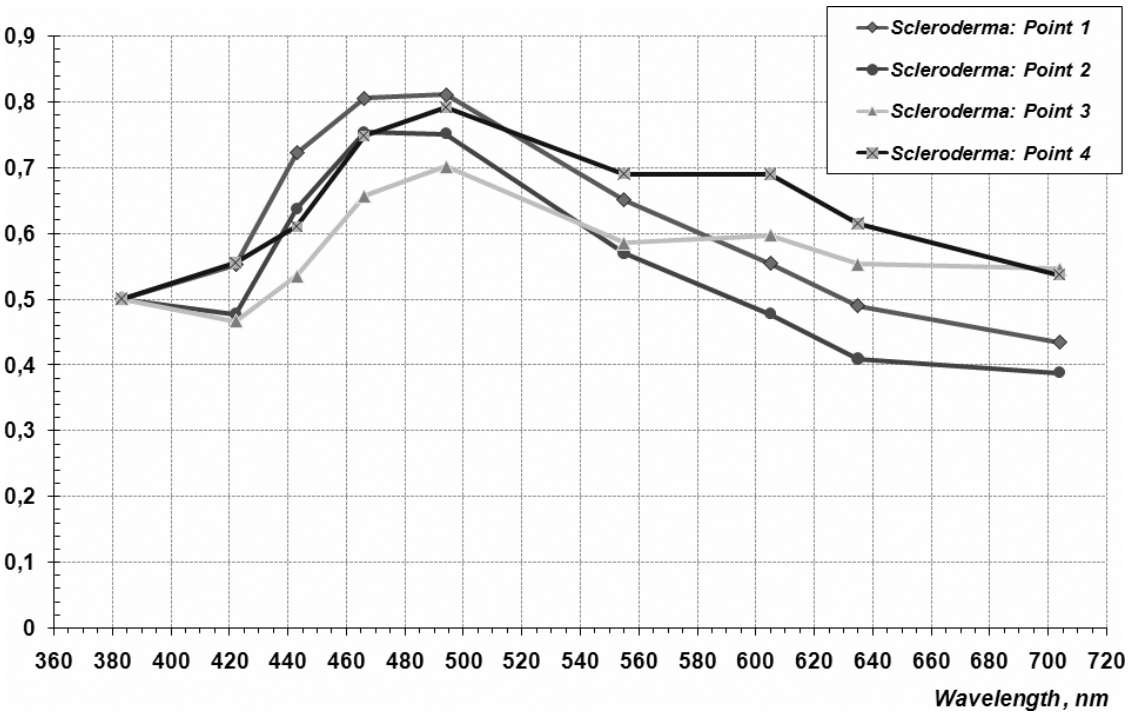
On the contrary, collagen contrast values close to those in healthy people (Fig. 5(b)) with a relatively high contribution of the collagen fluorescence to the general spectra are characteristic for scleroderma (Fig. 5(a)). Although, according to Ref. 7, collagen synthesis and degradation balance dysfunction with general increase of collagen concentration is reported in patients with scleroderma.

Thus, analyzing the fluorescence absolute intensity and contrast coefficients of separate fluorophores their concentrations cannot be precisely estimated at the moment. We suppose that the dependence of fluorescence intensity from fluorophore concentration is not only nonlinear, but also is nonmonotonic, i.e., theoretically at some concentration of the substance the regional maximum of the fluorescence amplitude will be observed. At concentrations higher or lower than this value around the region the fluorescence will be lower. Therefore at present we cannot locate this maximum concentration (which also depends on the temperature, media absorption and scattering indexes as well as on its phase and topological heterogeneousness). We cannot correctly identify a correlation of fluorophore's concentrations at various fluorescence intensity levels of these fluorophores *in vivo*.

It is possible that scleroderma could be the case of high collagen turbidity of the skin when the higher the concentration, the lower the signal. It is also possible that this fact is the consequence of the associated increased concentration of some other absorbing chromophores (for example, bilirubin). And as a last resort, the blood filling of tissues' microcirculation vessels contributes the most to the complexity of fluorophore's concentration measurements *in vivo*. Not only the spectrum of blood absorption in the optical waveband is very nonlinear and includes a collagen fluorescence waveband, but it has different values at various blood oxygenation levels as well. Therefore, taking parameters of blood microcirculation and oxygenation of the given tissue region into account is crucial for the *in vivo* fluorescent data interpretation.



(a)



(b)

Fig. 5. (a) Scleroderma tissues fluorescence spectra. (b) Coefficients of fluorescent contrast for different tissues in a patient with scleroderma for the spectra in Fig. 5(a).

4. Conclusions

The present study shows the possibility of laser spectral *in vivo* diagnostics of collagen fluorescence changes in scar tissue and some types of BCC. Scar tissue can be characterized with higher collagen fluorescence and corresponding contrast coefficients lower than normal. Lower values of collagen contrast coefficients are observed in most BCC. Fluorescent contrast coefficient of collagen can be slightly different in patients with scleroderma at generally high fluorescence amplitude. Differences in density, homogeneousness, blood filling and spectral characteristics for different human skin regions are typical even for normal tissue. Therefore it is crucial to learn how to take the influence of all these factors on resulting spectra into account for correct fluorescent tissue diagnostics *in vivo*. In our opinion, an exact mathematical model precisely describing nonadditive and nonmonotonic character of the dependence of optical spectra from concentrations of all influencing fluorophores and chromophores is required for quantitative evaluation of the collagen concentration in tissues on the basis of the *in vivo* fluorescence data.

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