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ALTERATIONS IN AUTOFLUORESCENCE SIGNAL FROM RAT SKIN *EX VIVO* UNDER OPTICAL IMMERSION CLEARING

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For the first time, the changes in autofluorescence spectra of *ex vivo* rat skin have been experimentally investigated using the combination of fluorescence spectroscopy and optical immersion clearing. The glucose, glycerol and propylene glycol solutions were used as clearing agents. The optical clearing was performed from the dermal side of skin imitating the *in vivo* injection of clearing agent under the dermal layers. In this contribution, the common properties of autofluorescence variation during optical immersion clearing were determined. The tendency of autofluorescence signal to decrease with reduction of scattering in tissue was noticed and discussed in detail. However, the differences in the shape of spectral curves under application of different clearing agents showed that optical clearing affects the autofluorescence properties of tissue differently depending on the type of clearing liquid. The results obtained are useful for the understanding of tissue optical clearing mechanisms and for improving techniques such as fluorescence spectroscopy.

Keywords: Autofluorescence spectra; fluorescence spectroscopy; rat skin; tissue optical clearing; clearing agents.

1. Introduction

Fluorescence spectroscopy is widely used for skin investigations such as estimation of skin photodegradation, determination of the areas with melanin content, estimation of skin erythema extent and pigmentation cancer diagnostics, etc.[1–3] The main limitation of this technique is the rather shallow penetration depth of optical radiation in biological tissues, caused by their optical heterogeneity. The shape of spectrum of emitted fluorescence may also be noticeably distorted because of the strong scattering in tissue. The tissue optical clearing method allows transient reduction of scattering within the optically heterogeneous tissues. The implementation of the method is based on the injection into the tissue of immersion chemical agents (glycerol, glucose, trazograph, propylene glycol, etc.), which influence the local optical properties of bulk tissue. Presently, there are three known mechanisms which take place in tissue during optical immersion clearing: (1) the matching of the refractive indices of scattering centers and ground substance; (2) dehydration, i.e., the loss of water by intracellular space, which leads to tighter package of the scattering particles and an increase in the ground refractive index[4–6]; (3) reversible dissociation of collagen fibers.[7–9] However, there is yet no final fundamental understanding of the mechanism of the tissue optical clearing. That is why the combination of the tissue optical clearing and fluorescence spectroscopy methods could be very helpful for a full understanding of tissue optical immersion

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clearing mechanisms and for increasing the diagnostic opportunities of fluorescence spectroscopy.

In this contribution, we use the combination of these two techniques (optical clearing and fluorescence spectroscopy) in order to predict the alterations in autofluorescence properties of biological tissues under optical immersion clearing. At present time, no scientific reports using the combination of these methods for investigation of tissue autofluorescence behavior are known to the authors. Previously, the influence of optical clearing effect on the registered fluorescence signal from the artificial object, placed under the skin was investigated.[10] A fluorescent film was placed under the skin *in vitro* and *in vivo*. The immersion agent was applied on the surface of skin. Then the fluorescence intensity emitted by the fluorescent film was measured. The rehydration of the samples was performed using the physiological solution after 20 minutes of immersion agent application. Finally, it was found that the intensity of the registered signal from the artificial object has increased during the essential scattering decrease. However, after rehydration of the samples, the opposite effect was observed: the fluorescence signal from the artificial object decreased, when the immersion clearing agent was replaced by physiological solution. In Ref. 11 the optical clearing was performed on the bulk sample of tissue. Based on the registration of fluorescence signal from red fluorescent protein, distribution of viable tumor cells inside the sample was obtained using optical emission tomography. The authors in Ref. 12 observed experimentally overweak chemiluminescence of artificially-generated sources through the pig skin *in vitro*. The aim of our work is to investigate the influence of immersion optical clearing on autofluorescence properties of rat skin *ex vivo* as well as to understand the mechanism of tissue optical clearing, and check if the usage of the combination of tissue optical clearing method with fluorescence spectroscopy can potentially improve the possibilities of autofluorescence diagnostics.

2. Materials and Methods

Experimental measurements of autofluorescence and reflectance spectra of skin samples during their optical immersion clearing were carried out using fiber-optic multichannel spectroanalyzer Lesa 5-med (Fig. 1). For the excitation of autofluorescence spectra, the nitrogen laser (1) providing radiation at the wavelength of 337 nm was used. For

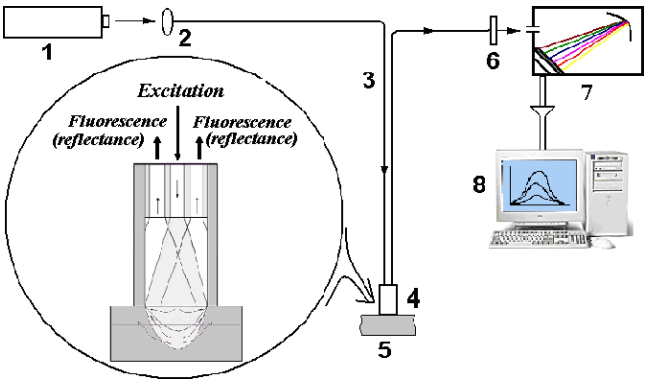

Fig. 1. Scheme of experimental setup. 1 — light source (halogen lamp, N_2 laser); 2 — lens; 3 — fiber-optic bundle; 4 — holder positioner; 5 — skin sample; 6 — filter; 7 — polychromator Lesa 5-med; 8 — personal computer.

recording of reflection spectra, the halogen lamp was used as a light source. The collecting lens (2) coupled the laser beam to the tip (3) of illuminating optical fiber. The illuminating fiber enters the bundle of fiber-optic probe trough Y-splitter and was in contact with the surface of the tissue sample (rat skin *ex vivo*) (5). The tip of fiber optical probe (4) was fixed in direct contact with the tissue sample surface with the aid of specially designed holder. The fluorescent or reflected light from the skin sample was collected by the detection fibers (six fibers, located around the central illuminating fiber of optical probe), and guided to the polychromator (7). The detection was carried out by a linear CCD array and analyzed on the personal computer (8).

All *ex vivo* autofluorescence and reflectance measurements were performed on the rat skin samples. Aqueous solutions of 50% glycerol (by volume), 40% glucose and 60% propylene glycol solutions were used as clearing agents. In this work we imitated *in vivo* tissue clearing under subcutaneous injection of clearing liquids. The registration of diffuse reflectance and autofluorescence spectra was performed from the epidermal surface. The geometry of the experiment is presented in Fig. 2. The skin sample (1) was stretched and fixed on the cored cylinder (3) by dermal surface of skin inside of the cylinder using thin rubber bands. After that the fiber-optic probe (5) from the spectrometer Lesa 5-med was fixed using the holder and placed in direct contact with epidermal surface of skin. Then the spectra registration was performed. Spectra were registered every 3–5 minutes for one hour. The fresh skin samples were taken from rats directly before the experiment. Hair and

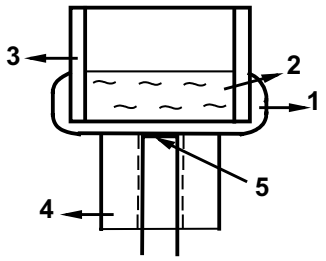

Fig. 2. The measuring cell for registration of the temporal kinetics of skin autofluorescence spectra *ex vivo* under continuous unilateral contact with clearing agent. 1 — skin sample; 2 — immersion agent; 3 — cylinder; 4 — fiber holder; 5 — tip of the optical fiber from Lesa 5-med.

hypodermic adipose layer were removed from the skin. Then the skin was cut into pieces and kept in physiological solution before the beginning of experiment. The sample sizes were about 3 cm×2.5 cm in order to keep the reserve of skin for its fixing on the cylinder. The cylinder sizes were 1.5 cm in diameter and 2 cm in height. The thickness of each sample was about 1 mm.

3. Results and Discussion

The data obtained for temporal dynamics of the autofluorescence intensities recorded from rat skin *ex vivo* during optical immersion clearing are presented in Fig. 3. For all cases corresponding to different clearing liquids, it is seen that on average the skin was cleared for 30–40 minutes. The clearing process for some agents is more effective than for the others. The shape of the curve for

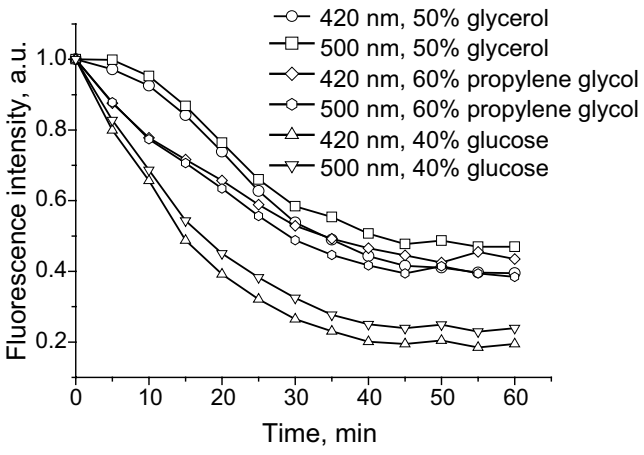

Fig. 3. Normalized temporal dynamics of autofluorescence spectra of *ex vivo* rat skin under clearing by 50% glycerol, 60% propylene glycol and 40% glucose solutions at 420 and 500 nm wavelengths.

glycerol solution is different at the beginning stage of clearing process. These dissimilarities could be attributed to different influence of biocompatible liquids on the tissue. Another suggestion is that decreasing of autofluorescence intensity is not only due to the matching of refraction indices within the tissue. There could be other mechanisms which can take place in the tissue during optical clearing. In order to answer this question, relative autofluorescence spectra were analyzed. Additionally, due to the same reason, the reflectance spectra were recorded under the same experimental conditions.

For better understanding of what happens in tissue under conditions of our experiment, the light propagation in the skin was analyzed, considering only the fact that clearing agents influence the matching of refractive indices and that the scattering will be reduced with time. The skin was cleared from the dermis side and the autofluorescence and reflectance spectra were registered from the epidermis side. The different wavelengths of the visible radiation are absorbed in the different layers (depths) of the skin. The longer the wavelength, the more deeply light penetrates. Thus, the long-wave radiation will be reflected mostly by deep layers. Due to this fact, at the beginning of the experiment, light reflected from the upper layers was mostly registered. The detectors collected mainly the short-wave light due to scattering. When the tissue was cleared, the scattering coefficients became equal for all layers. As a result, light started to penetrate through tissue and the probabilities of light to be reflected for long- and short-waves became equal. That is why the curve smoothing for different wavelengths was observed when the skin was cleared. In contrast to reflectance, the influence of clearing on the collected autofluorescence signals has different behavior (see illustrative example in Fig. 4). Here we have the excitation radiation with the wavelength of 337 nm. The propagation of UV-radiation in tissue is shallow and the upper layers give the main contribution to the autofluorescence signal. At the beginning of the experiment, there were no alterations in autofluorescence signal. A small increase of the curve in the first 10–15 minutes (see Fig. 3) was observed because the clearing took place in the bottom layers of skin. As soon as the clearing agent reached the upper layers, the radiation started to pass through the bottom layers. Bottom layers also gave a small contribution to the autofluorescence signal. Thus, after some time, when the scattering was reduced, the bottom layers

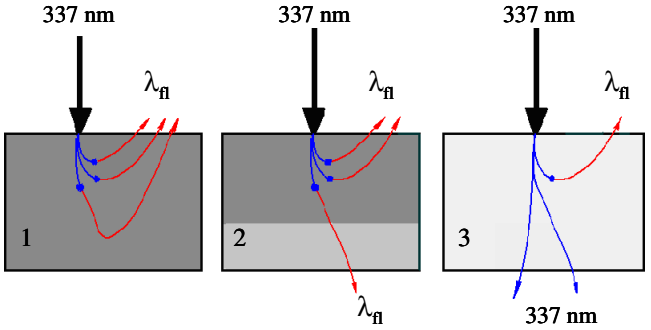

Fig. 4. The mechanism of autofluorescence signal decreasing under tissue optical clearing. 1 — native sample of tissue; 2 — the tissue sample after some period of optical clearing; 3 — cleared sample of tissue.

started to transmit light. Finally, the autofluorescence signal was decreased as the result.

The major objective of our research was to control the changing of the shape of autofluorescence spectra during the clearing process. For convenient analysis of spectral changes, in reflectance and fluorescence signals of the spectral dependencies, which we called relative spectra, were built. We took the registered spectra for two selected moments of time t_1 and t_2 ($t_1 < t_2$) and then the values $I_{\text{rel}}(\lambda)$ were calculated using the next expression: $I_{\text{rel}}(\lambda) = I_{t1}(\lambda)/I_{t2}(\lambda)$, where $I_{t1}(\lambda)$ is the intensity measured at wavelength λ at the timepoint of t_1, I_{t2} is the intensity at the wavelength λ at the timepoint of t_2. During the clearing process, both reflectance and fluorescence intensities are decreased; as a result, the values of relative intensities in spectra are always not less than one. The changes in autofluorescence spectra during the clearing process are presented in Fig. 5. The curves for all investigated clearing agents have the common behavior: the spectrum corresponding to the final stage of clearing is flat for all wavelengths, i.e., the decrease of autofluorescence intensity is uniform for the whole wavelength range. However, the initial stage of the process changes with the clearing in a blue range.

This difference between the shape of the autofluorescence curves for different clearing agents at the initial stage of optical clearing is clearly seen (Fig. 5). This could be explained by the fact that the immersed clearing agents can differently affect the tissue properties, and probably different mechanisms take place in tissue under application of different agents. Similarly depending on the clearing agents, the autofluorescence properties of tissue might be changed differently at the initial stage of clearing. In order to answer this question, the

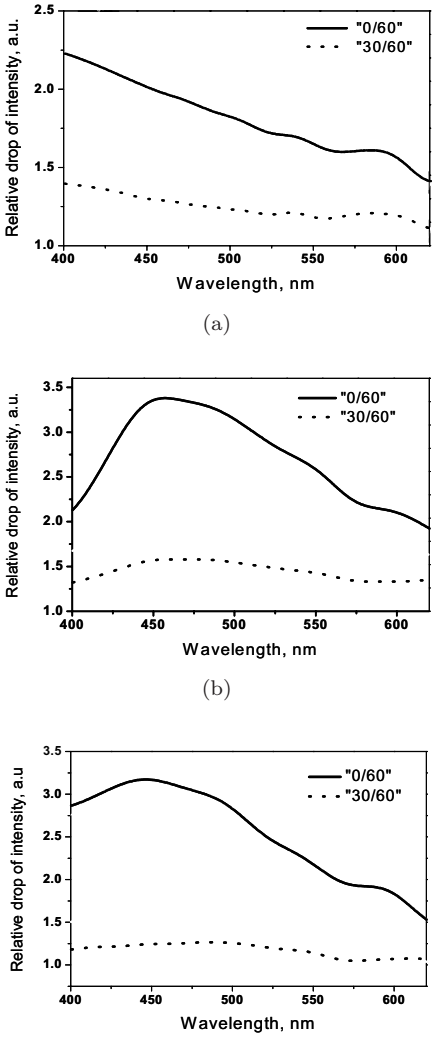

Fig. 5. Relative autofluorescence spectra of rat skin *ex vivo* for different periods of optical clearing by: (a) 50% glycerol; (b) 60% propylene glycol; (c) 40% glucose solutions. The inset indicates the time relations.

reflectance spectra of rat skin *ex vivo* recorded under the same conditions were analyzed (Fig. 6). Comparing the reflectance spectra for different clearing agents, the following can be noticed: if we suppose that the shape of reflectance spectrum of skin can be described by the expression α/λ^{β}, then the exponent for relative spectrum will be equal to the difference between the exponents of the reflectance spectra at the beginning and at the end stage of clearing process. Taking into account the spectra presented in Fig. 6, the decreasing of exponent under clearing by propylene glycol exceeds the same values for glucose and glycerol solutions. This can be evidence of the fact that the propylene glycol more effectively matched the refractive

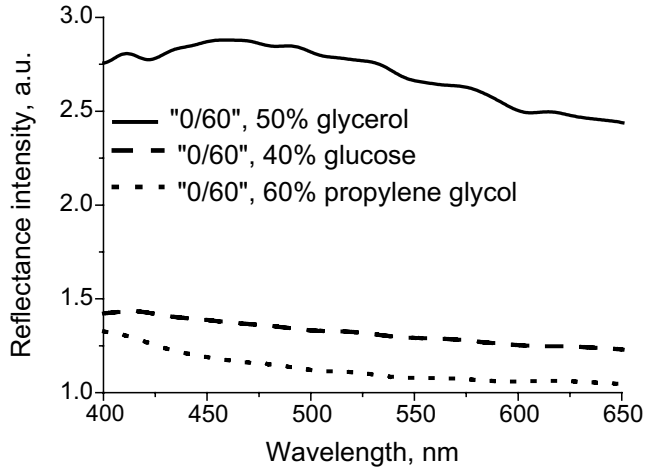

Fig. 6. Relative skin reflectance spectra for one-hour clearing by 50% glycerol, 40% glucose, and 60% propylene glycol solutions.

indices of scattering centers and base substance. If we assume the presence of any types of scatterers in skin with different physico-chemical properties, then we can conclude that the propylene glycol results in the matching of refractive indices of the fraction of small scatterers.

The spectral reflectance curve for glucose solution has a similar shape with the propylene glycol, but it has high reflectance intensity. The spectral curve for glycerol is smoother and it has the highest intensity in comparison with the other curves (Fig. 6). According to the above-mentioned exponential dependence, such difference for glycerol solution could be related to its influence on the scattering particles with bigger sizes. It is not excluded that the previously proved existence of the collagen dissociation mechanism during optical clearing by glycerol solution[7–9] can explain this differential curve behavior using glycerol solution. From the autofluorescence and reflectance spectral dynamics for propylene glycol solution, it is seen that the clearing process is very slow in comparison with the clearing by other agents. This could be attributed to the presence of groups of fluorophores whose fraction of fluorescence is changed differently under tissue optical clearing. Another observation is that in reflectance signals, it is possible to distinguish the spectral selection for different clearing agents. For glycerol solution, the decreasing of signal takes place in all spectral range. The reflectance signals of glucose and propylene glycol solutions demonstrate the presence of similar dependence as in the case of autofluorescence spectra for these clearing agents. At the early stages of clearing, the intensity is decreased at the short-wave part of spectrum; at the end of clearing process, the intensity is decreased more uniformly throughout all wavelength range.

4. Conclusion

We have investigated the alterations of autofluorescence spectral properties of *ex vivo* rat skin under application of clearing agents such as 40% glucose, 50% glycerol, and 60% propylene glycol solutions. The clearing agents were applied from the dermal side of investigated skin samples. The utilized method of relative spectra allows comparison of common alterations in autofluorescence spectra under optical immersion clearing. From the results obtained it is possible to conclude that the spectral changes are different at different stages of clearing process. The differences were found in relative spectra between different types of clearing agents. The common regular trend in autofluorescence spectra for glucose, propylene glycol and glycerol solutions is as follows: at the initial stage the clearing process is more effective in the blue part of the spectrum, and at the final stage the rate of clearing equalizes. However, we observed the different shapes of curves for different clearing agents. We suppose that it is related not only to the matching of refraction indices within the tissue, but also to the presence of other processes which take place in the tissue during clearing when observed with fluorescence spectroscopy. For the full confirmation of this fact, additional investigations on autofluorescence properties of tissues under optical immersion clearing are necessary.

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