Research on lymphedema by method of high-resolution multiphoton microscopy

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Abstract. In this paper results of *iv-vivo* measurements for healthy volunteers and people, with verified diagnosis of lymphedema, obtained with two-photon tomography with fluorescence lifetime imaging microscopy (FLIM) are presented. The papillary layer of the skin was analyzed at a depth of about 100 μm. The purpose of this study is to evaluate the lifetime of autofluorescence in the papillary dermis of healthy tissue and with the initial stage of lymphedema. In the course of the study, a small redistribution of autofluorescence lifetimes was observed for healthy volunteers and people with lymphedema disease. The research was carried out on the equipment of MPTflex (JenLab GmbH).

1. Introduction

Lymphedema is associated with impaired functioning of the lymphatic system. This disease can be both congenital and acquired as a result of surgical interventions. Disease progression caused by abnormal accumulation of tissue fluid with a high protein content leads to proliferation of fibrous connective tissue, fat hypertrophy and inflammation [1, 2]. Consequently, it provokes the abnormal functioning of the immune apparatus. At the moment, treatment of this disease is complicated by many factors, including the complexity of accurate clinical assessment of disease [3]. Treatment of chronic swelling of the limbs is a grand problem because there is no established cure. There are many methods that can correct limb swelling. These methods provide prescription for effective treatment [4]. However, current X-ray (CT), lymphoscintigraphy, ultrasound imaging and so on allow often not the desired resolution for the diagnosis of early-stage disease. The problem solving is the non-invasive image of single intratissue cells and even intracellular compartments in different tissue depths. With the advent of modern multi-photon lasers, it became possible to conduct measurements of subcutaneous fat in-vivo without adversely affecting the subject of the study [5, 6]. Moreover, with using technology for determining the lifetime of autofluorescence, it became possible to divide objects by the lifetime. It is known, that multi-photon tomography (MPT) provides images with submicron spatial resolution and 200 μm depth penetration during *in vivo* skin analysis. In article [7] authors presented list of the excitation and emission wavelengths for major skin fluorophores. Multiphoton tomography is a novel non-invasive multidimensional imaging technology with subcellular resolution used along with dermatoscopy, ultrasound and confocal reflection microscopy in tissue analysis. Hence, MPT has advantage at both, sensitive detection of endogenous fluorophores and active

extracellular matrix components like collagen in second harmonic generation (SHG) mode. Multiphoton tomography enables functional imaging of deep-tissue cells and their cellular compartments. For instance, in the research [8] the fluorescent mitochondrial reduced coenzyme NAD(P)H was detected. These findings provide information about the intracellular redox state and modifications of the cellular metabolism. Recently it was shown that with fluorescence lifetime imaging microscopy (FLIM) it is possible to isolate capillaries in the papillary layer of the skin [9].

In the work [10] the femtosecond laser produced fluorescence in biological tissues is explored. The fluorescence properties of femtosecond laser treated biological samples were systematically characterized by means developed a combined single-photon confocal and two-photon fluorescence spectroscopy system. It records time- and spectral-resolved single- and two-photon fluorescence emissions at the same location of sample. These fluorescence properties served as a basic guide for efficiently differentiating the femtosecond laser treated tissue from surrounding intact tissue through the single-photon confocal or two-photon microscopy. In the paper [11], it was demonstrated that it is possible to estimate the FLIM data after processing the original signal, this allows to reduce the scatter in the evaluation of the lifetimes of autofluorescence.

Thus, multiphoton microscopy, based on two-photon fluorescence or second harmonic generation, has a great potential for non-invasive evaluation and monitoring of morphological structures and functional states of living tissues [6, 12].

In addition, multidimensional FLIM data provides an opportunity to estimate metabolism of tissue. The first detailed study of differentiation of basal carcinoma cells from normal keratinocytes was given using multispectral multiphoton fluorescence lifetime imaging. To visualize multidimensional FLIM data phasor plot and principal component analysis (PCA) is used. Also, development of a diagnostic classification algorithm with all of the spectroscopic and cellular morphology parameters, requires dimensionality reduction techniques [13] to avoid a "curse of dimensionality" problem. PCA and its modifications are the state-of-the-art multidimensional data analysis technique [14-17].

2. Experimental Part

In this work, two groups of patients were examined: healthy volunteers and patients with lymphedema disease undergoing treatment at the ANI "Research Institute of Microsurgery of the Tomsk Scientific Center of the Siberian Branch of the Russian Academy of Medical Sciences". The study protocol was approved by the institutional committee of the Research Institute of Microsurgery, and each participant signed the "Information consent" for participation in the study. The study involved a group of patients with stage II lymphedema (target group) aged 23 to 68 years (n = 8) and a group of healthy volunteers aged 19 to 65 years (n = 8). The first group included 2 patients with primary lymphedema of the lower extremities, 2 patients with secondary lymphedema of the upper limbs (after axillary lymphadenectomy due to breast cancer) and 4 patients with secondary lymphedema of the lower limbs, caused by various causes, namely extensive skin burns (1 patient), after trauma (1 patient) and after surgical treatment of cervical cancer in combination with X-ray therapy (2 patients).

Images of subcutaneous fat were obtained on a two-photon microscope MPTflex (Jenlab GmbH, Germany). The working wavelength of the pump laser was 760 nm. The papillary layer of the skin was studied at a depth of about 100 μm. The corneal layer of the skin was chosen as the initial depth of the laser position. Each measurement was carried out using the FLIM mode and a special software package "Becker & Hickl". The original data was a 128 × 128 × 256 pixel matrix, the image size was 75 × 75 μm with a lateral resolution of about 1 μm and a longitudinal resolution below 2 μm. The data was analyzed using the SPCImage application (Becker & Hickl) and Python.

In this paper, the lifetime of autofluorescence (AF) was analyzed. The AF signal is usually estimated using two exponential approximations as follows

$$F(t) = a_1 e^{-t/t_1} + a_2 e^{-t/t_2} \, , \qquad (1)$$

where a_1, a_2, t_1, t_2 are the amplitudes and lifetimes of the AF signal. The average lifetime t_m is given by a formula:

$$t_m = \frac{t_1 a_1 + t_2 a_2}{a_1 + a_2} \qquad (2)$$

and this value is often used to analyze FLIM data. The spatial distribution of t_m values for healthy tissue and lymphedema tissue is shown in figure 1.

3. Results and Discussion

This study is focused on research in region with maximally possible depth (using our device without addition substances, i.e. the papillary dermis). We estimated depths about 100 μm for different location with similar structures. It is important to note lifetime on this depth it cannot very accurate since the intracorneocyte autofluorescence can be distorted, primarily by that of the SHG signal from the collagen fibres and fluorescence from the epithelial cells. However, in the present study, it was estimated not by the exact values of the lifetimes, but by averaged over a significant number of examples. So we can rely on the distribution of lifetimes.
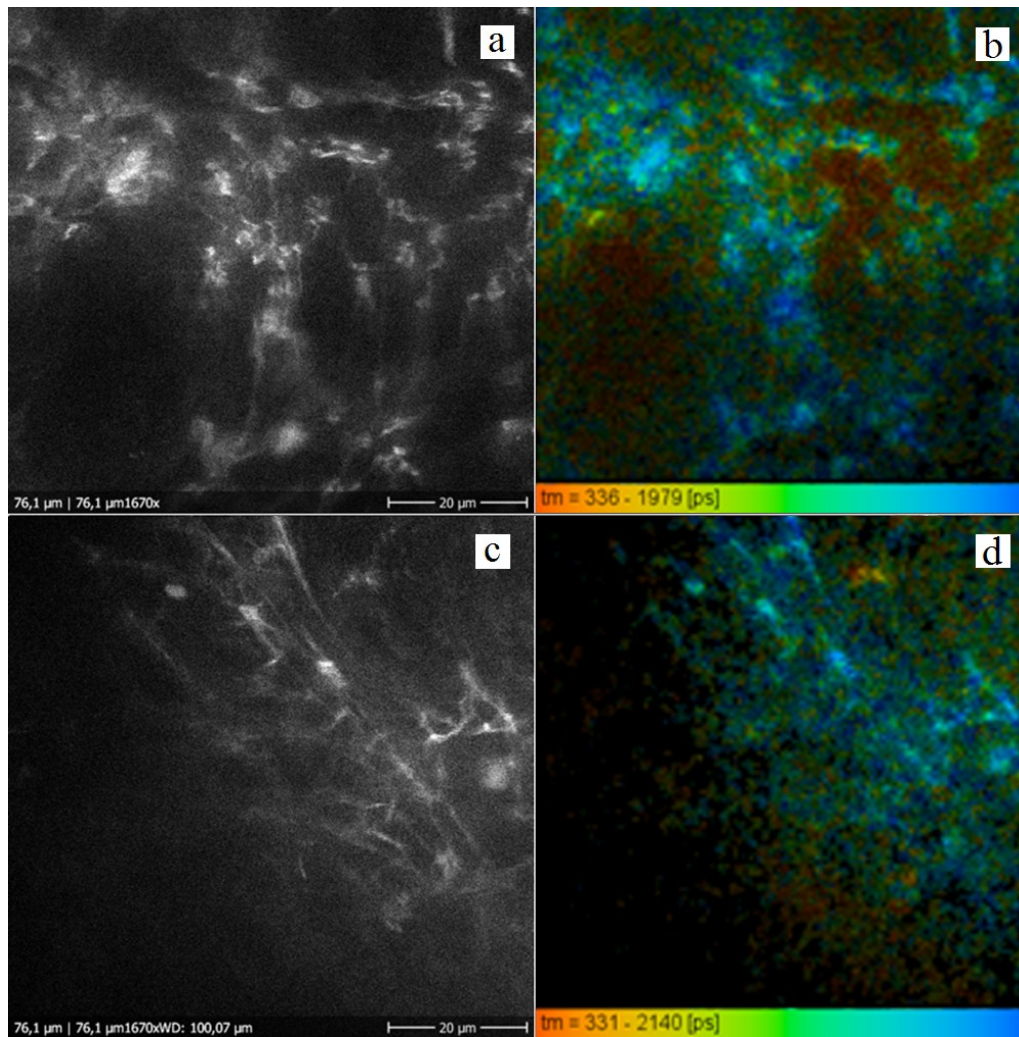

Figure 1. An example of FLIM analysis for lymphedema tissue (the upper row) and for healthy tissue (the lower row): AF images (a, c), t_m images (b, d).

The spatial distribution healthy of tissue and lymphedema tissue is presented in figure 1 demonstrated the values of intensities (a) for healthy, (c) for patients with lymphedema disease and lifetime t_m values for healthy (b) and patients (d). The above images give an understanding of what kind of data we are working with and show that despite the structural difference of the images, we can talk about a sufficiently close lifetime of autofluorescence.

Averaged over 20 images, t_m value for healthy tissue and lymphedema tissue is shown in figure 2. A small variation of the t_m value takes place, but the overall distribution of the t_m is same for healthy tissue and lymphedema tissue. Thus, we can conclude that content of fluorescent substances, including elastin, are similar for groups under study, so the main difference between them connected with spatial variations of collagen structure.

In this paper, we compared a set of similar areas for healthy and patients with lymphedema, it was assumed that the distribution of life-times would differ, since changes in the structure of collagen could have an effect on the lifetime of the environment.
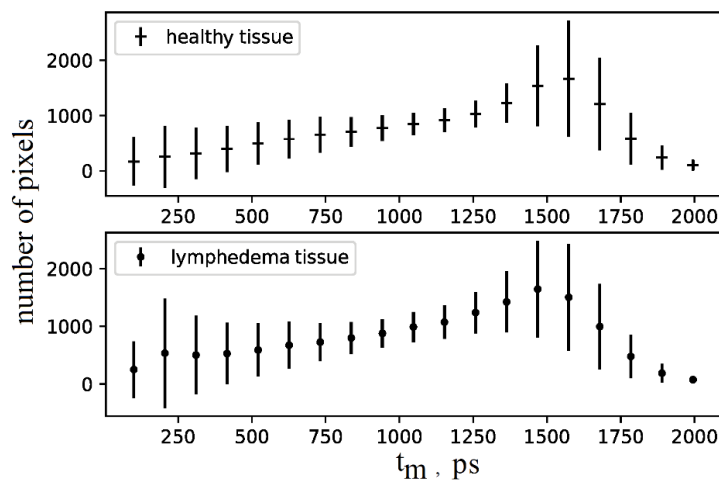

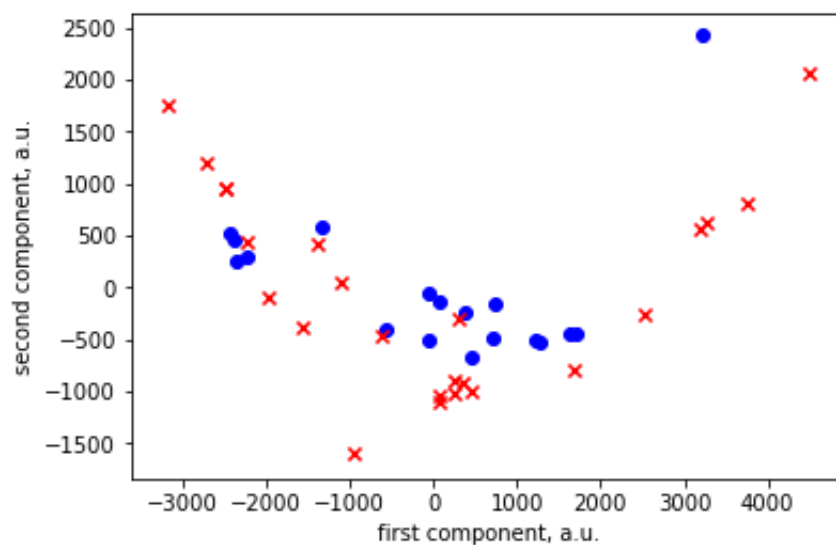**Figure 2.** Mean value and standard deviation for lifetime t_m.

Figure 3. Two first principal components for lifetime t_m obtained for health and lymphedema patients.

Figure 3 shows two first principal components for autofluorescence decay curves for healthy people and people with lymphedema. With the selection of the main components for the decay curves of autofluorescence, an increased standard deviation can be noted for patients with the lymphedema disease. Moreover, in the space of the main components for healthy people, we observe a compact set of points. Thus, we can talk about the applicability of this method for the task of separating healthy and lymphedema people. Besides, since PCA reveals patterns in the data, from figure 3 we can deduce that there are identical areas for healthy people and people with lymphedema. But for people with lymphedema, data deviation is higher, so our hypothesize is that this method allows us to perform classification of healthy and tissue with lymphedema.

4. Summary

During the research, redistribution of autofluorescence lifetimes for healthy volunteers and people with lymphedema disease was noted, however, the main component composition in similar parts of the papillary dermis did not change significantly. This allows us to make the assumption that as a result of the development of the disease, structural changes occur that do not significantly affect the component composition of the tissue.

Acknowledgments

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French P, Chu A, Stamp G and Dunsby Ch 2012 Multiphoton Multispectral Fluorescence Lifetime Tomography for the Evaluation of Basal Cell Carcinomas *PLoS One* **7(9)** e43460

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