Improvement of the multiphoton fluorescence microscopy images quality using digital filtration

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ABSTRACT

In our study we used rank-order filter, the emissions filter on the base of the criteria of Pearson, Gaussian filter and median filterfor improving the is fluorescence lifetime imaging microscopy (FLIM) data. The data obtained with the FLIM technology are the distribution with a pronounced peak, while during measurement the peak value is measured with an error. According to the analysisthe Gaussian filter is more useful to improve quality of FLIM data.Spatial filtering allows to reduce the noise component, obtained in the course of measurements, including reduction the influence of the individual bursts. Filtering in time scale allows to determine a peak value of intensity more accurately.This research was carried out using the equipment of Tomsk Regional Common Use Center of Tomsk State University.

Keywords: second harmonic generation, autofluorescence, FLIM, FRET, data analysis.

1 INTRODUCTION

Multiphoton laser microscopy (multiphoton laser tomography, MPT) provides instantaneous imaging of living skin at the cellular and subcellular levels. MPT can use the autofluorescence of fluorophores of intrinsic tissues and second harmonic generation (SHG) from matrix tissue components, such as collagen, thereby providing functional and structural visualization of uncolored biological tissue.[1]

Apowerful tool for studying microobjects in vivo and ex vivo is fluorescence lifetime imaging microscopy (FLIM). This method provides a quantitative determination of the spatial distribution of sample fluorescence (the lifetime of the map). FLIM demonstrates great potential in biomedical imaging, materials science and chemical analysis because it gives information about a time dynamics of fluorescence radiation over the sample area and can represent the redox status of skin cells.[2,3,4]

In practice, FLIM data is often used not in one point but in some region of interest(information for each pixel in region of interest are combined in one pixel with more number of photons). An example of FLIM data (by Becker &Hickl) is presented in the Figure 1.

The fluorescence resonance energy transfer method (FRET)allows to estimate the inter-molecular interaction. To measure protein interactions, proteins are labeled with two dyes of different absorption and emission spectra. The emission band of the first dye (donor) must to overlap the absorption band of the second (acceptor). If the distance between donor and acceptor is less than a few nanometers, energy can transfer directly from the donor to the acceptor. The result is a decrease in the fluorescence decay time of the donor. The intensity of energy transfer, that is, the decrease in the decay time, is an indicator of the distance between the donor and the acceptor.[5]

The combination of the fluorescence lifetime imaging microscopy (FLIM) and phasor approach (phasor-FLIM) provides a simple method for histopathology analysis and can greatly improve the accuracy of diagnosis. Phasor approach can be used to visualize the spectra and decay curves. The phasor plot is a graphic representation of the raw FLIM data. In each pixel, the fluorescence decay curve can be written in the phasor plot.

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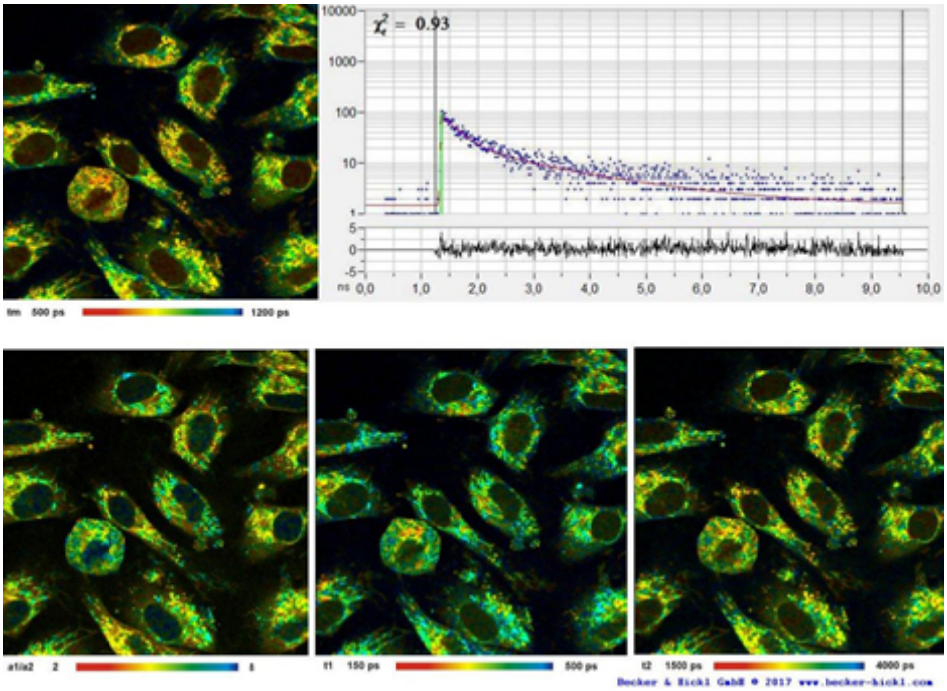


Figure1. FLIM data fitting in the application SPCImage (by Becker &Hickl) .[6]

The decay curve is converted to a point with the coordinates g and s, whereω – modulation frequency,is given in expression (1):[7]

$$g(\omega) = \frac{\int_0^\infty I(t)cos(\omega t)}{\int_0^\infty I(t)} \qquad s(\omega) = \frac{\int_0^\infty I(t)sin(\omega t)}{\int_0^\infty I(t)} \quad . \tag{1}$$

The most important feature of this analysis is that it is fast and provides a graphical representation of the measured curve.[8]

A multi-photon tomography makes it possible to obtain detailed images of human skin in-vivo. Information about time dynamics of fluorescence radiation over the sample can be obtained using these data. In addition, information about the skin condition can be obtained with the help of data on SHG and autofluorescence (AF), including the level of aging of the skin. To assess the aging of the skin, various layers of the skin (epidermis, papillary layerand dermis) are studied and specific indices are estimated, for example, the ratio of collagen to elastin.

The studies[9,10] give examples of using the SAAID index to estimate the skin aging. The results of the mentioned works demonstrate a sufficiently high variance in the indices for subjects of the same age. As an example, Figure 1 shows that the data obtained during the measurement for short time intervals have a large enough spread, which can be explained by a number of factors: influence of neighboring areas, errors in the course of the detector, features of the object of research. The influence of these factors can be reduced by solving with a sufficient number of deviations, temporal and spatial filtration.

In the study of the dermis were observed "dark" region, without giving the luminescence channel at the second harmonic generation. These areas can be caused by the hair follicle, capillaries.[12, 13] For example, research[13]shows the results of studying the capillaries in papillary layer data using the FLIM. It is established that the capillaries are visible on autofluorescence and give a distinctive glow. In such studies there is the problem of evaluating decay time autofluorescence in small areas, as the equipment cannot produce in sufficient detail the timing of photons in a limited region of space. Thus, data processing of this type becomes important.

All these methods are based on laser radiation, in view of the complexity of the processes of generation of radiation, as well as its detection, the data obtained may be of unsatisfactory quality. In particular, the noise in individual pixels can effect on the resulting image, as well as artifacts occurring in certain areas of the image. These problems can lead to false

classification. In our studies, spatial filters were used to analyze the experimental data, including a rank filter and time filters: Gaussian and median.

2 MATERIALS AND METHODS

In this study, the measurements of dermis were carried out on the MPTFlexmicroscope by Jenlab (Germany). This equipment is allowed to work with people. The study involved a group of volunteers (n=10) at the age of 22 to 68 years. For each subject, 10 images were recorded at a depth of about 100 μm.

Images of the elastin and collagen fluorescence were acquired simultaneously with 2 photomultiplier tube detectors on the volar forearm. The image size was 75μm×75μm and images were recorded on a 512×512 pixel matrix. The analysis was done with software package "Becker&Hickl".

The rank filter based on the sliding window. The values in the window were defined by user and the k-element of the array was selected. Rank filter is a generalization of other filters, for example, morphological filters (dilatation, erosion), median filter.[11]

Also the sliding window of the specified size is used in theGaussian filter. Convolution is performed with the Gaussian function (the size is the same as the window size) inside the window. The two-dimensional form of the Gaussian function is given below:

$$f(t) = \frac{1}{2\pi\sigma^2} e^{-(x^2+y^2)/2\sigma^2}. \quad (2)$$

3 RESULTS

To reduce the spread in the values of the aging index of SAAID and the ratio of collagen to elastin, a Gaussian filter was applied. To estimate the quality of filtration, the standard deviation was used. At each level, 32x32 size areas were selected and for each region the collagen to elastin ratio, SAAID index were calculated. Further, for each subject, the root-mean-square deviation for the values was estimated. After this, Gaussian filtration was applied to all the data obtained, and then the average value and the spread were again analyzed.

Table 1.Estimating age using the ratio of collagen and elastin and SAAID

	Original data		Data after Gaussian filtration		Data after Median filtration	
	Young volunteer, collagen to elastin ratio / SAAID	Aged volunteer, collagen to elastin ratio / SAAID	Young volunteer, collagen to elastin ratio / SAAID	Aged volunteer, collagen to elastin ratio / SAAID	Young volunteer, collagen to elastin ratio / SAAID	Aged volunteer, collagen to elastin ratio / SAAID
Mean value	1.62/0.33	2.01/0.45	1.63/0.33	2.00/0.44	1.63/0.33	1.99/0.43
First quartile	1.32/0.21	1.83/0.38	1.32/0.21	1.84/0.38	1.23/0.23	1.33/0.38
Third quartile	2.10/0.43	2.42/0.53	2.01/0.40	2.38/0.52	2.05/0.40	2.36/0.50

The results show a reduction in the noise component for each volunteer. The spread is reduced by smoothing of the image that is reducing the noise value due to weighted averaging within the window region.After applying of the median filter to the measurements, we obtain better noise removal of MPT data. The spread in the values of the collagen to

elastin ratio becomes smaller, which indicates a more qualitative separation.All these results have a confidence interval less than 0.05 compared with the aged volunteers group (this interval was obtained using U Test Mann-Whitney).

In these studies, for the areas of interest, the distribution of photons in time (region of 10x10) was considered. Figure 2 shows the example of FLIM data obtained as a result of summing all the photons in the area under consideration.

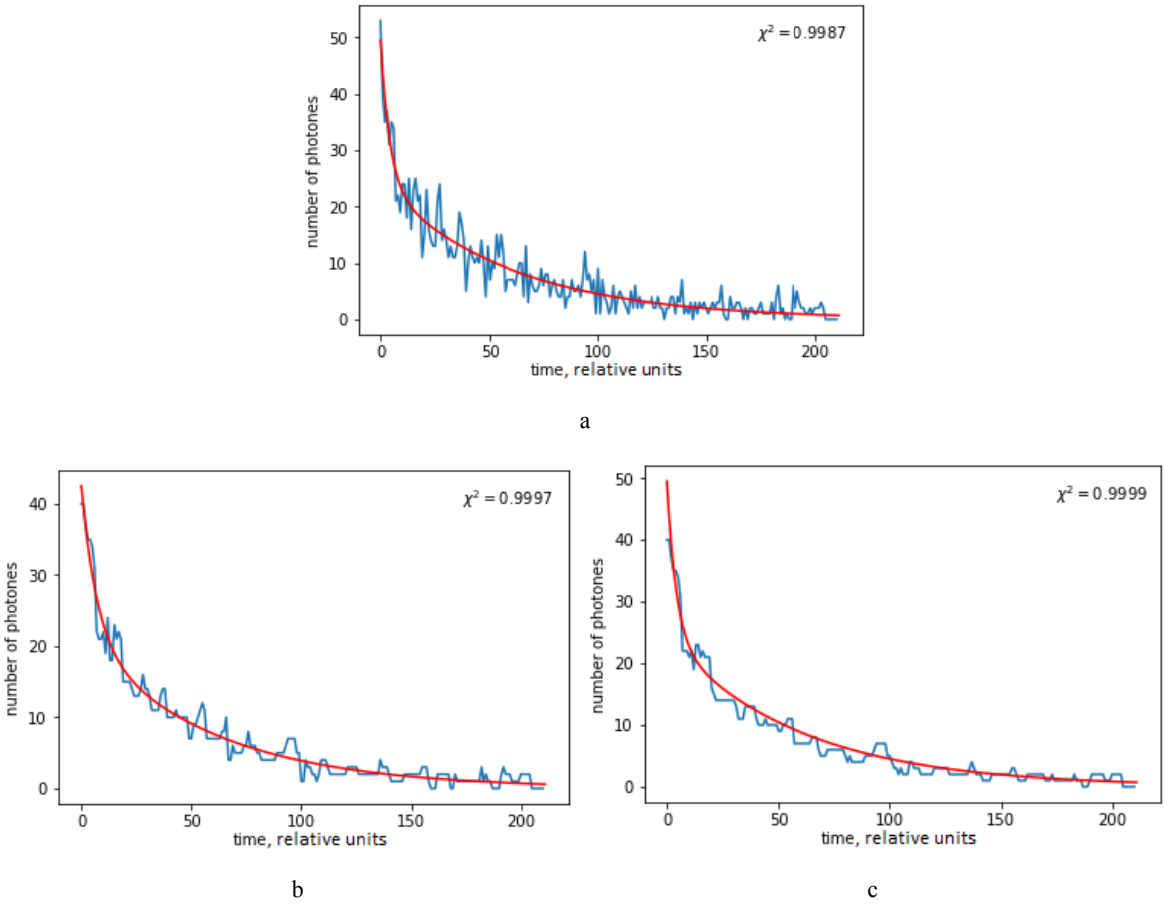
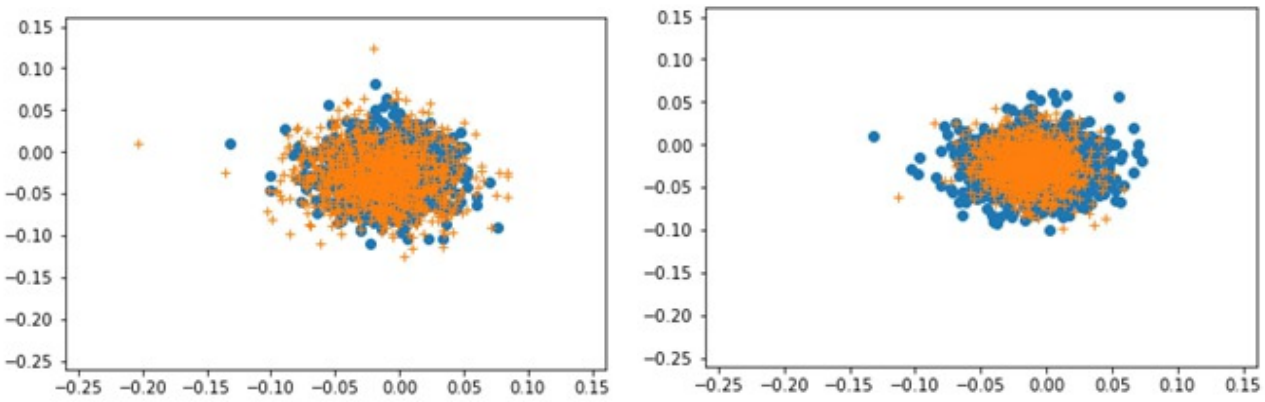


Figure 2.The decay of flioserscence signal: a) total value of photons in the 10x10 region, for different time intervals, b) data after applying the rank filter with the window 4, rank 2 c) data after applying the rank filter with the size 3, rank 7.

The phasors plots (see Fig.3) were constructed using the formulas(1). The modulation frequency was chosen in view of the wavelength of the laser used (760nm). In this case, using a filter with a window size of 7 and a parameter of k = 3 gives better filtering of data and reduces the spread, not only on the original FLIM data, but also transformed by the phasor approach.



a b

Figure 3.Image of FLIM data on phasor plot: a) blue marks depict original data, orange marksdepictdata after rank filter with k = 2 and rank = 4 b)blue bluemarksdepictoriginal data, orange marksdepictdata after rank filter with k = 3 and rank = 7

4 CONCLUSION

We useddigital filters to reducethe noise from data obtained by MPT, MPT-FLIM. The results obtained with MPT (data from AF and SHG) show that filtration allows to decreaserandom variations in the collagen to elastinratio. It is shown that the median filter allows to reduce noise more qualitatively, since the data after filtering is separated more qualitatively, the first and third quartiles are closer to the average value. It is also true for FLIM data. These results are obtained not only in the case of approximation of the damping curve, but also in the phase space using the phasor approach.

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