Analysis of Collagen Fibers from Second-Harmonic Generation Images of Skin

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Abstract: *Multiphoton microscopy is an important noninvasive technique for the in situ imaging and characterization of biological structures as skin. The second harmonic generation (SHG) microscopy has been extensively used in the study of collagen fibers. In the present study, we report the development of a nonlinear optical microscopy system for tissue imaging by both SHG and two-photon excited fluorescence using a Ti:Sapphire femtosecond laser. The system was designed to allow in vivo and ex vivo imaging. The study of the collagen fibers is a powerful way to track the evolution of skin conditions like the photoaging or wound healing. To characterize and semi-quantify the collagen structure, a method for the analysis of SHG images was used. The mean diameter of the collagen fibers was calculated through the two-dimensional autocorrelation function of the image and a novel technique to quantify the ordination of the fibers is presented.*
Keywords: *Multiphoton microscopy, SHG microscopy, collagen fibers, autocorrelation function, anisotropy.*

Introduction

Multiphoton microscopy is an important noninvasive technique that allows the investigation and *in situ* characterization of structures in the skin. Compared to the confocal microscopy, it presents the advantages of a deeper tissue penetration due to the longer wavelength of the illumination source and lower photodamage [1,2].

This technique presents lower resolution than confocal microscopy but shows a higher specificity for some biological structures. The high intrinsic second harmonic generation (SHG) of collagen fibers [3] has been extensively used in the SHG microscopy for the analysis of mechanical properties of tissues. The analysis of the SHG images of collagen fibers in the skin usually takes into account their mean diameters but one important parameter to be studied is the ordination of the fibers, especially for wound healing and cosmetics.

An automated method for the analysis of sizes distribution of collagen fibers using the two-dimensional autocorrelation function (ACF) has been presented by Raub *et al.* [4] and a detailed study of the fibers ordination using the discrete Fourier transform (DFT) and other techniques has been presented by Matteini *et al.* [5].

The commercial nonlinear microscopes design does not allow *in vivo* imaging and this prevents the investigation of important phenomena in complex live systems over time. In this paper we present the development of a nonlinear microscope designed to allow simultaneous SHG and two-photon excited fluorescence (TPF) imaging of *in vivo* and *ex vivo* samples. We also propose a novel method for the analysis of the ACF of SHG images of collagen fibers and for the study of the fibers ordination.

Material and Methods

The developed multiphoton laser-scanning microscope follows the scheme shown in Figure 1.
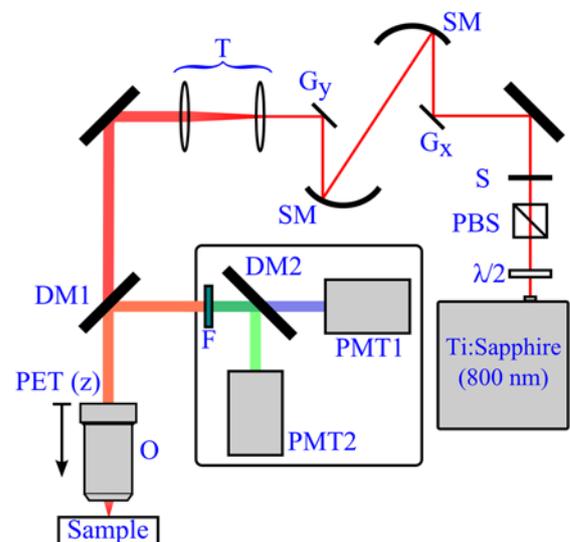
Figure 1: Schematic setup of the microscope.

A mode-locked Ti:Sapphire laser emitting with maximum intensity at 800 nm was used. The power is controlled by a system composed by a half-wave plate ($\lambda/2$) and a polarizing cube (PBS). The xy scanning is done with the aid of two spherical mirrors (SM) and two galvanometer mirrors (G_X and G_Y). The beam is expanded by a telescope (T) composed by two lenses and then focused at the sample by a water immersion objective (O, Olympus XLUMPLFN20X) attached to a

piezoelectric transducer (PET) for displacement in z direction.

The signal from the sample is collected by the objective and filtered (F) to remove the signal from the backscattered laser light by the dichroic mirror (DM1). The signal is then separated in two spectral regions (SHG and TPF) by a second dichroic mirror (DM2) and delivered to two photomultiplier tubes (PMT1 and PMT2). The presented design allows the acquisition of the SHG and TPF images simultaneously. A more detailed description of this experimental setup is given elsewhere [6].

The ACF $A(\vec{\delta})$ of a two-dimensional real function $f(\vec{r})$ is defined by the following equation:

$$A(\vec{\delta}) = \int d^2r \, f(\vec{r}) f(\vec{r} + \vec{\delta}). \qquad (1)$$

For an image constituted by $M \times N$ pixels, the discrete autocorrelation function can be calculated by:

$$A_{i,j} = \sum_{m=1}^{M} \sum_{n=1}^{N} f_{m,n} f_{m+i,n+j}, \qquad (2)$$

where $f_{m,n}$ is the intensity of the pixel in the n-th row and m-th column of the gray level image.

In practice, the Wiener-Khinchin theorem allows us to reduce processing time by computing the autocorrelation matrix by means of discrete Fourier transformations [4].

The ACF of an image carries important statistical information. For nonperiodic functions, its value will decay asymptotically to a minimum when $f(\vec{r})$ and $f(\vec{r} + \vec{\delta})$ become statistically independent [7]. The characteristic size of the shift $\vec{\delta}$ necessary to achieve this situation is related to the sizes of the structures shown on image and is calculated by fitting analytical functions on the ACF matrix. Raub *et al.* have shown that Gaussian distributions are good functions to fit the ACF of SHG images of collagen fibers and that the sizes obtained by this procedure are related to the fibers width.

The ACF is not only influenced by the sizes of the fibers in the image but also by the ordination of these fibers. We observed that the ACF of anisotropic images is not a monotonic decay in all directions but a distribution with major axis in the direction of the main anisotropy axis as illustrated in the Figure 2.
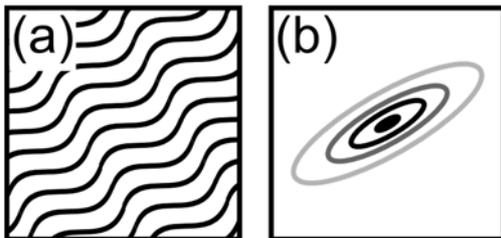
Figure 2: Illustration of an anisotropic image (a) and its autocorrelation function (b).

To quantify the aspect of the autocorrelation function e define the anisotropy tensor $\overleftrightarrow{Q}$, shown in Eq. 3.

$$\overleftrightarrow{Q} = \int d^2r \, A(\vec{r}) \, (\vec{r} \cdot \vec{r} \, \mathbb{I} - \vec{r} \otimes \vec{r}), \qquad (3)$$

where $\mathbb{I}$ is the identity tensor. This tensor has the exact same form of the inertia tensor of a rigid body with the density of mass being substituted by the autocorrelation function $A(\vec{r})$.

$\overleftrightarrow{Q}$ can be written in terms of dyadic products as follow:

$$\overleftrightarrow{Q} = \int d^2r \, A(\vec{r}) \, (r^2 \hat{x}_i \hat{x}_i - x_i x_j \hat{x}_i \hat{x}_j), \qquad (4)$$

with $x_1 \equiv x$, $x_2 \equiv y$ and the Einstein summation convention being used.

The off-diagonal elements of $\overleftrightarrow{Q}$ indicates that the original x and y axis are not symmetry axis of the ACF. To find the symmetry axis it is necessary to diagonalize $\overleftrightarrow{Q}$ by finding the unitary matrix S that satisfies:

$$S^\dagger \overleftrightarrow{Q} S = \overleftrightarrow{D}, \qquad (5)$$

where $\overleftrightarrow{D}$ is the diagonal tensor of eigenvalues of $\overleftrightarrow{Q}$.

It is important to note that $\overleftrightarrow{D}$ is the exact same tensor than $\overleftrightarrow{Q}$ but written in the basis of its eigenvectors which are, in this case, the vectors that define the axis of symmetry of the ACF. Thus, the main direction of anisotropy is easily obtained by the eigenvector ($\vec{v}_1$) with the lowest eigenvalue (λ_1) and we define the anisotropy factor R as:

$$R = 1 - \frac{\lambda_1}{\lambda_2}, \qquad (6)$$

where λ_2 is the largest eigenvalue associated with the eigenvector $\vec{v}_2$. The factor R admits, by construction, values between 0 and 1. Lower values of R are related to more anisotropic images while higher values are related to highly ordinated fibers.

Now, instead of fitting a function directly on the ACF data, we extract two profiles from the ACF in the direction of the vectors $\vec{v}_1$ and $\vec{v}_2$ and fit a single exponential decay of the form:

$$y = a. e^{-x/d} + b. \qquad (7)$$

For each one of the profiles, there will be different parameters calculated. The parameter d calculated in the direction of $\vec{v}_2$ correlates with the diameter with the fibers. All the calculations were performed with the software GNU Octave [8].

Results

SHG images from fresh biopsy of Wistar rats skin were taken with the presented microscope. All animal

procedure was approved and carried out in compliance with the guidelines of the Ethical Principles in Animal Research (Ethics Committee on Animal Experimentation of School of Medicine of Ribeirão Preto, University of São Paulo, Brazil, process n° 062/2009). The images were analyzed by the methods described and the size obtained from the ACF is compared to the mean diameter measured manually in the image. One example of the images and its autocorrelation function are shown in Figure 3.

It is possible to see through the ACF that the SHG image of collagen fibers presents a certain degree of anisotropy. Measuring the mean fibers diameter in the image we obtain a narrow distribution around $2.5\ \mu m$.

The main direction of anisotropy was identified by the presented algorithm as $95°$ in respect of the horizontal direction. The diameter of the fibers calculated through the fitting of an exponential decay on the profile obtained in the direction perpendicular to the main axis of anisotropy was of $d_2 = 2.70\ \mu m$ while the same parameter calculated through the fitting on the profile extracted along the major axis was of $d_1 = 4.19\ \mu m$. The anisotropy factor was of $R = 0.57$.
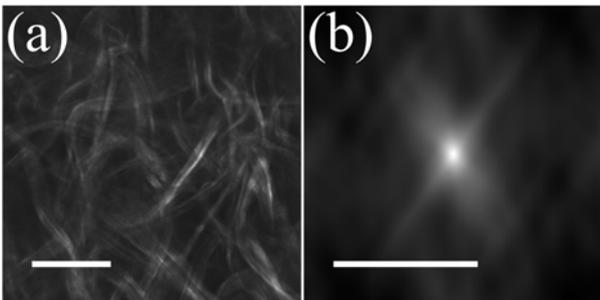
Figure 3: SHG image of rat skin showing the collagen fibers (a) and correspondent autocorrelation function (b). Bars: $25\ \mu m$.

The other images composing the set are part of a z-stack performed by scanning the same region of the skin and varying progressively the depth of focal plane by displacing the piezoelectric transducer (PET, Figure 1). The same analysis was carried out with the whole set of images and similar results were obtained to all those in which the fibers could be clearly observed.

Discussion

The presented microscope allowed us to obtain images of the collagen structures of rat skin using the SHG of these fibers with good resolution. The images were analyzed through the calculation of the ACF which allowed us to estimate their mean diameter.

As expected, the mean diameter correlates with the parameter d obtained from the perpendicular direction of anisotropy with good approximation.

Further experiments with *in vivo* imaging are being carried out and we aim to study the progress of the discussed parameters over time in samples with different skin conditions.

Conclusion

The calculation of the ACF is clearly a powerful tool to characterize structures such as the collagen fibers. We have shown that it is possible to obtain, with good accuracy, statistical quantities as the mean diameter of the structures through a careful analysis of the function. We also could successfully evaluate the anisotropy of the images through the proposed method.

A possible adjuvant analysis for the presented methods is the calculation of the entropy of the image and of its Fourier transformation.

Acknowledgements

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