Characterization of third-degree burned skin by nonlinear microscopy technique

Moisés Oliveira dos Santos[a], Vitor Bianchini Pelegati[b], Carlos Lenz Cesar[b], Paulo Roberto Correa[a], Telma Maria Tenório Zorn[c], Denise Maria Zezell[*a]
[a]Instituto de Pesquisas Energéticas e Nucleares, IPEN – CNEN/SP, Av. Lineu Prestes 2242 - Cidade Universitária, São Paulo, SP Brasil 05508-000
[b]Universidade Estadual de Campinas, Instituto de Física Gleb Wataghin, Depto. de Eletrônica Quântica, Cidade Universitária Zeferino Vaz s/n, Barão Geraldo, Campinas, SP – Brasil 13083-970
[c] Universidade de São Paulo, Ed. Biomédicas I, Av. Lineu Prestes 1524, Cidade Universitária, São Paulo, SP Brasil 05508-900

ABSTRACT

Nonlinear microscopy imaging technique enable take both images of collagen fibers in dermis through second harmonic generation (SHG) signal and elastic fibers by two-photon emission fluorescence microscopy (TPEFM). These techniques are the most commonly used technique for turbid and thick tissue imaging and also to image biological samples which presents highly ordered structural proteins without any exogenous label. The objective of this study is characterizing dermis of third-degree burned skin by TPEFM and SHG technique. The modelocked laser (Spectra Physics) source used in this study with pulse width of approximately 100 fs at 80 MHz was directed into a multiphoton microscope using a laser scanning unit (Olympus Fluoview 300), mounted on an inverted confocal system microscope (Olympus IX81), with focusing objective (40x, NA = 1.30). The samples were obtained from Wistar rats, male, adult. One dorsum area was submitted to burn caused by vapour exposure. The biopsies obtained were cryosectioned in slices of 20 μm width. Selected area of interface between the injured and healthy subdermal burned skin were imaged by TPEFM and SHG technique. Two different autofluorescence signals are observed as a function of excitation wavelength. The autofluorescence observed at 760 nm and 690 nm suggest components of extracellular matrix at differents depths. In SHG images, collagen fibers are visible. According to the images obtained, these methodologies can be used to characterize dermis of burned tissue as its healing process with reduced out-of-plane photobleaching and phototoxicity.

Keywords: SHG, TPEFM, third-degree burn skin imaging, dermis, fibroblasts, collagen, elastin, fibronectin, extracellular matrix

1. INTRODUCTION

Determination of burn wound depth is important for the assessment of injury and proper burn management. Thermal trauma causes an extensive loss of soft tissue. Thus, to regenerate the full-thickness wound close by subsequent wound contraction and ephitelization[1]. Various stages appear on healing process. One important phase is the proliferative, which is characterized by fibroblast migration, deposition of the extracellular matrix and formation of granulation tissue[2]. To characterize third-degree burned skin the nonlinear microscopy offers advantage because images possess high contrast and high deep penetration, these nonlinear microscopy techniques include: Two-photon emission fluorescence microscopy (TPEFM), and second harmonic generation (SHG)

TPEFM and SHG are the most commonly used technique for turbid and thick tissue imaging and also to image biological samples which presents highly ordered structural proteins without any exogenous label[3,9]. In two-photon emission of fluorescent molecules, two-photon are absorbed in same quantum event, as determined by the Heisenberg uncertainty principle, within a time-scale of process of 10^{-16} - 10^{17} s.[4,5] The interaction occurs if the sum of the energy of the two photons is equal to the energy required for conventional excitation with one-photon absorption. Two-photon typically use long wavelength, in range of near-infrared (700 – 1000 nm), which enable deep optical tissue imaging, because NIR light is less scattered than visible light[6]. With complementary information detailing biological system structure, SHG is a coherent process which photons of an intense laser light, passing through nonlinear structure

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generates another photon with twice the optical frequency (i.e. half the wavelength) in the medium, as opposed to two-photon fluorescence[6]. The intensity of signals varies by technique. In SHG, the intensity is slightly dependent on excitation wavelength, while the signal of two-photon emission is strongly influenced by the wavelength of laser light and spectral absorbance of structure. The aim of this study is characterize dermis of third-degree burned skin by TPEFM and SHG technique.

2. METHODOLOGY

2.1 Laser System and Multiphoton Microscope System

The laser system used in this study is a Mai Tai HP (Spectra Physics, Santa Clara, CA), pumped femtosecond Titanium:Sapphire laser (Millennia®, Spectra Physics, Santa Clara, CA), pulse width of approximately 100 fs at a 80 MHz repetition rate. For TPEFM the laser was operating at 766 nm and 690 nm. For SHG was 793 nm. The Multiphoton Microscope System consists of a laser scanning unit (Olympus Fluoview 300), mounted on an inverted confocal system microscope (Olympus IX81) with oil immersion objective (40x, NA = 1.30), for focusing the excitation beam and for collecting of the backward signals.

2.2 Tissue Preparation

After approval of Animal Research Ethics Committee, the samples were obtained from Wistar rats, male, adult, sacrified by lethal dose of anesthetic. In dorsum of each animal one area were submitted to burn, with 8 mm of diameter, caused by 90 ^{o}C water vapour exposure, during 8 s. The third-degree burn area was biopsied after third, fifth, seventh and fourteenth days post sacrifice. The burn skin sample collected at each day were bath in isopentane for cryopreservation and storage at a bottle of liquid nitrogen (-196 ^{o}C). Before imaging burned skin samples were cryosectioned perpendicular to the epidermal layer comprising a transverse cross section of the epidermal and dermal layers, in slices of 20 μm width, and mounted on glass slides and covered with coverslip for viewing. For TPEFM/SHG images no exogenous labels were used.

2.3 Image measurement method

The Fast Fourier Transform (FFT) was applied to two-dimensional images to investigate the structural alteration of collagen orientation in dermis, which is performed with the module of the image analysis software ImageJ (version 1.43u, National Institutes of Health, USA). To estimate collagen orientation, the calculus of width/height ratio of the zeroth-order maximum in the generated power plot of the two-dimensional images of SHG was used[7,8]. The collagen orientation was calculated by (1 – W/H), where: W = width, and H = height of the length of the arcs in the power plot. The collagen orientation index varies in range of 0 to 1. Parallel collagen orientation results in an orientation index tending to 1. Perfectly randomly oriented collagen tending to 0.

3. RESULTS

Selected area TPEFM and SHG images of interface between the injured and healthy subdermal third-degree burned skin are shown in Figure 1. Using multichannel mode the image of subdermal burned skin can be imaged. As can be seen from Figure 1, each line represents one day of sacrifice, and there are three columns of images. The column a the TPEFM with excitation wavelength of 690 nm, the elastin and fibroblast components of dermis can be isolated and blue color-coded. In column b, the TPEFM with excitation wavelength of 760 nm, elastin and fibroblast components in different depth is green color-coded. The column c, the SHG excited with wavelength of 793 nm, show collagen of subdermis imaged, and red color-coded. Monitoring wound healing process the image show in blue and green colors-coded the distribution and content of ECM components. As the days go on the signal progressively improve at this depth, providing visual identification of increase components secreted by cells present in the ECM.

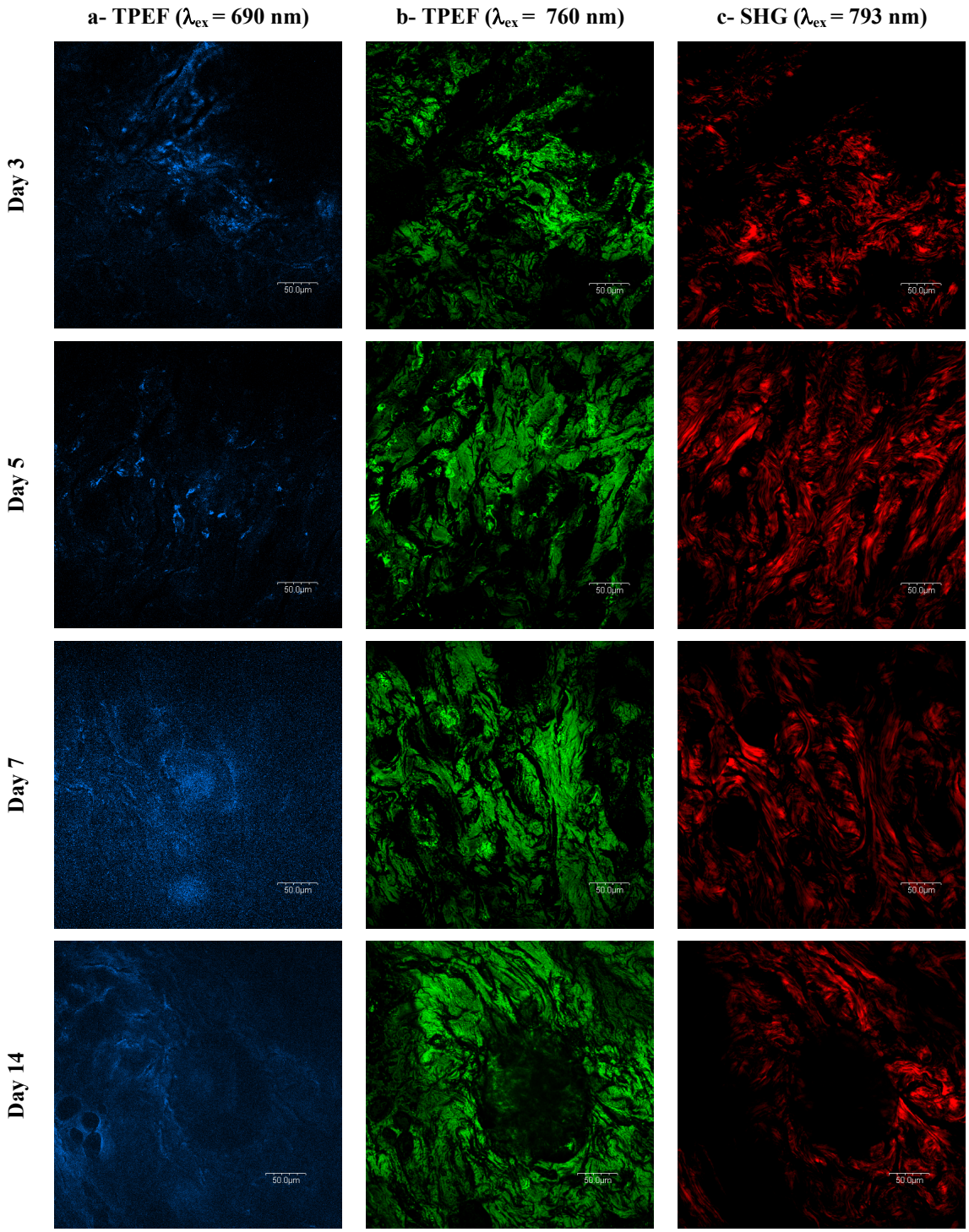
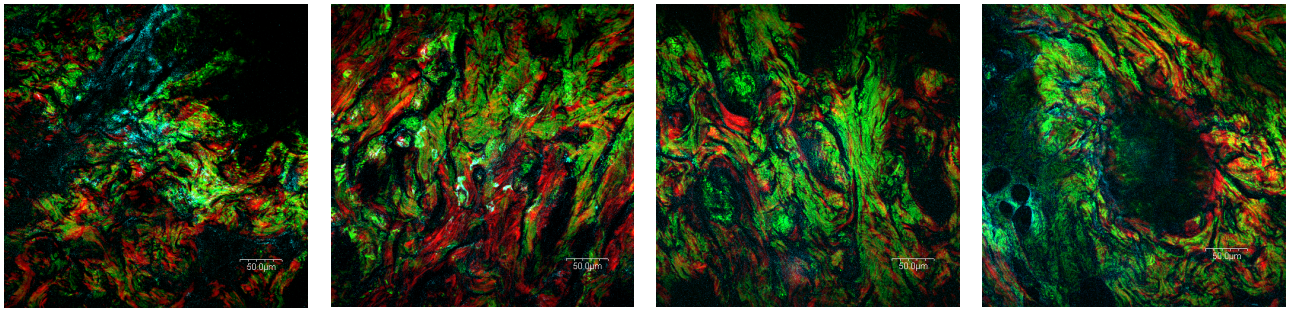


Figure 1. Images of perpendicular section to the epidermal layer with 20 µm thick. Column (a) represent TPEFM excited with wavelength of 690 nm; column (b) represents TPEFM excited with wavelength of 760 nm; and column (c) represents SHG excited with wavelength of 793 nm. The fluorescence of TPEFM comes from ECM components and SHG signals comes from collagen mesh. Scale bar = 50 µm.

The figure 2 shows the overlay of the TPEFM and SHG images allowing the observation of ECM components between collagen fibers at third, fifth, seventh and fourteenth days. Structural details of dermis can be characterized due to high-contrast TPEFM/SHG images. The collagen fibers (red color-coded) are visible in details in third and fifth day and progressively covered by ECM components in following days, forming a mixed of colors.



Day 3 **Day 5** **Day 7** **Day 14**

Figure 2. Overlay of TPEFM and SHG images. The images were obtained by overlaying three channels, showing overlap of structures and components of dermis. Scale bar = 50 μm.

Collagen synthesis is the sign of fibroproliferative phase of wound healing and beginning of renewed structural integrity. Collagen is sensitive to cell-mediated regulation, particularly in healing tissues that forms an extensive cable-like network that provides increase tensile strength to the wound. To calculate the collagen orientation index, three regions of each image was used to estimate orientation index. The results, given in Table 1, showed no conclusive differences of collagen orientation in dermal region during the healing process of burned skin. The results implied that perfectly randomly oriented collagen is present in dermis and didn't achieve the degree of order that is found in normal or unwounded dermis.

Table 1. Collagen orientation index.

Burn skin	Number of areas	Mean	S.D.
day 3	3	0,34	0,13
day 5	3	0,30	0,18
day 7	3	0,42	0,02
day 14	3	0,31	0,09

4. DISCUSSION

The results presented here have served to investigate the evolution of wound healing process along of time, supporting the fact that TPEFM and SHG microscopy is effective to analyses third-degree burned skin. We observed that ECM components appearing in minor intensity of signal in beginning of healing process and increase with time. This may be due to fibroblasts that restore mechanical stability of wounds by producing collagen, fibronectin, elastin, and proteoglycans[11,12]. The morphological structure of collagen as can seen change observing images, because plays an important structural role in skin, interstitial tissue and basal laminae and its synthesis is the hallmark of the fibroproliferative phase of wound healing and beginning of renewed structural integrity. We recognize that collagen orientation index no presented difference in results, may be due to scars restrain that collagen organization achieves the degree of order that is found in normal dermis, should be more research with larger amount of data to analyze. We

further note that nonlinear microscopy is a powerful method to characterize wound skin and analyses with other types of wound healing can be used.

5. CONCLUSIONS

According to the images obtained by TPEFM and SHG, these methodologies can be used to characterize dermis of burned tissue as its healing process. The signals acquired have the capability to better differences the endogenous proteins structures that originates the SHG and TPEFM. These techniques allow live cell imaging with reduced out-of-plane photobleaching and phototoxicity[2].

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