European Conferences on Biomedical Optics 2021 (ECBO) © OSA-SPIE 2021

Numerical Analysis on *ex vivo* Second Harmonic Generation Images of Collagen Structure of Unstained Basal Cell Carcinoma Sections

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Abstract: FFT analysis on mosaic SHG images of basal cell carcinoma skin sections of different subtypes is presented. This analysis combined with two-photon auto-fluorescence imaging might be useful for assessment of tumor borders.

1. Introduction

Basal cell carcinoma (BCC) is the most common malignancy in Caucasians [1]. Although BCCs rarely metastasize, local tissue degradation may lead to severe health and cosmetic damage and inoperability [2]. The gold standard in the therapy of BCC is surgical excision. However, BCCs often have poorly defined borders challenging complete excision [3]. Therefore, there is a demand for efficient imaging techniques for numerical evaluation of the tumor borders of BCC prior to and during surgery. Previously, second harmonic generation (SHG) nonlinear microscopy mosaic imaging was used to analyze collagen structure of BCC skin samples and increased collagen fiber length, decreased fiber angle and significantly higher fiber alignment of collagen fibers in BCC was found [4]. In this study, we carried out fast Fourier-transformation (FFT) analysis on mosaic SHG images from BCC skin sections of different subtypes (nodular, micronodular and invasive BCC). Our aim was to investigate if there are any differences in their collagen structure and to differentiate between subtypes according to the properties of collagen fibers, as well as to determine tumor borders. Our result shows that accuracy of FFT analysis highly depends on the unit cell size parameters (resolution, physical dimension of rectangular image portions) used for FFT. Furthermore, we found that collagen fibers around hair follicles give similar FFT results as fibers around tumor nests, which might hamper exact determination of tumor borders. Combination of FFT analysis with two-photon auto-fluorescence imaging, however, might offer a useful imaging tool for assessment of tumor borders of BCC.

2. Methods

4 pcs nodular, 4 pcs micro-nodular and 4 pcs infiltrative basal cell cancer samples were collected, formalin fixed and paraffin embedded, then 50 μm thick deparrafinized sections orthogonal to the skin surface were prepared from tissue blocks for SHG and TPEF imaging.

Nonlinear microscope images were captured by a commercial *Axio Examiner LSM 7 MP* laser scanning two-photon microscope (Carl Zeiss AG, Jena, Germany), with custom-modified detection optics for SHG imaging. For 2P excitation, a ~20 MHz repetition rate, tunable, sub-ps Ti:sapphire laser (*FemtoRose TUN LC GTI NoTouch*, R&D Ultrafast Lasers Ltd., Budapest, Hungary, see Ref. [5] for details) was used at an operation wavelength set to 800 nm. Violet (405/20 nm) and orange (590/40 nm) band-pass emission filters were respectively used to spectrally select TPEF and SHG signals. For focusing of the laser beam, a 20× water immersion objective (W-Plan – APOCHROMAT 20×/1,0 DIC (UV) VIS-IR, Carl Zeiss AG, Germany) was employed providing an imaging area of ~0.42 × 0.42 mm^2. Mosaic images were acquired by using a computer controlled, stepping motor driven X-Y positioning stage developed for tissue sections fixed on microscope slides (*Mosaic Positioning System*, R&D Ultrafast Lasers Ltd., Budapest, Hungary).

Fast Fourier Transformation (FFT) was carried out to measure the degree of organization and symmetry of collagen fibers [6]. FFT output images were converted to power plots for more accurate analysis [7]. As the eccentricity of the power plots reflects the arrangement of the collagen fibers, an ellipse was fitted to each power plot. In this study, we calculated collagen arrangement (CA) as the ratio of long axis/short axis of ellipse. A circular power plot reflects a normal skin sample where collagen shows an isotropic behavior, while an elongated power plot indicates parallelly oriented fibers [8]. FFT processing and image analysis were performed using a public domain image processing software (*ImageJ*). Individual (~0.42 × 0.42 mm^2) images were divided to 4 (2x2), 9 (3x3), 16 (4x4) or 25(5x5) even quadrangles (that we refer to as unit cells), for more precise spatial analysis. All unit cells with a total intensity