NONLINEAR OPTICAL MICROSCOPY FOR THE INVESTIGATION OF SKIN ALTERATIONS IN PSEUDOXANTHOMA ELASTICUM

PhD thesis outlines

Luca Fésűs

Clinical Medicine Doctoral School
Semmelweis University

Supervisor: Prof. Norbert Wikonkál, MD, PhD, DSc
Consultant: Róbert Szipőcs, PhD

Official reviewers: Erika Varga, MD, PhD
Barbara Molnár-Érsek, PhD

Complex Examination Committee:
Head: Prof. Tivadar Tulassay, MD, PhD, DSc
Members: Adrienne Vajda, MD, PhD
Balázs Kiss, MD, PhD

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Introduction

In the last few decades, due to the rapid technical development and the appearance of commercially available devices, nonlinear optical techniques have been implemented into dermatological experimental and clinical settings. Low energy laser sources operating in the near infrared spectral range are able to excite chromophores or structural elements that allows high spatial and temporal resolution imaging without any harm to the skin. Two-photon excitation fluorescence (TPEF) and second harmonic generation (SHG) can reach as deep as 400 μm below the skin surface, or with the use of skin biopsy sections, the whole cross section of the skin can be visualized *ex vivo*. With TPEF technique one is able to detect the keratin, elastin, melanin, NADH and FAD, whereas SHG is suitable to visualize non-symmetric molecules with high spatial organization, such as collagen. Pseudoxanthoma elasticum (PXE) is a rare, genetic metabolic disease that leads to ectopic mineralization and fragmentation of the elastic fibers, mainly in the skin, eye and blood vessels. Due to the subtle skin signs in the first years, patients are often only diagnosed, when severe ophthalmological or cardiovascular complications are already developed. Besides, phenotype is characterized by heterogeneity and determinants of severe organ manifestations are yet not known.

Our research group collaborates with the Femtosecond Lasers for Nonlinear Microscopy laboratory at the Wigner Research Centre for Physics. In our present work we investigated *ex vivo* skin sections from patients with PXE with TPEF and SHG techniques. Our results suggest that nonlinear microscopy is suitable for the diagnosis of PXE, and further characterization of dermal changes are possible with the use of a special staining. These findings can be used in the future for the diagnosis and monitoring of PXE patients.

Objectives

I. Experiment: To utilize *ex vivo* TPEF and SHG imaging in PXE

1. Is *ex vivo* NLM suitable to visualize histopathological alterations of elastin and collagen, and reveal calcium deposits in PXE skin sections orthogonal to the skin surface with TPEF and SHG techniques?

Diagnosis of PXE is currently based on histopathology and/or genotyping. Besides conventional hematoxylin and eosin staining, Weigert's elastic stain is used to show fragmented, shredded and clumped elastic fibres and von Kossa stain visualizes mineralized elastic fibers and calcium deposits in the mid-dermis. Sometimes, collagen fibre alterations are also visible. As elastin has strong TPEF signal and collagen emits SHG signal, these two

nonlinear optical methods are suggested for the examination various skin diseases. PXE skin sections were already examined with multiphoton techniques, but calcium deposits could not be visualized so far, that would be essential to set up the diagnosis. The purpose of our work was to examine the TPEF and SHG signal of *ex vivo* PXE skin sections orthogonal to the skin surface.

2. Do quantitative parameters of elastin and collagen fibres differ between healthy controls and PXE patients?

PXE is manifested in alterations in the mid-dermis. In PXE mineralization of the elastic fibres lead to their breakage and degradation. Although more aspecific, irregular diameter of collagen fibres and collagen flowers also have been described. Calcium cystals are deposited in the mid-dermis. These changes can be visualized by TPEF and SHG images. We aimed to quantify connective tissue changes with image analysis softwares.

3. How variable is the extent of calcification in the mid-dermis? Does this correlate with a possible soluble biomarker, PPi?

Plasma inorganic pyrophosphate (PPi) is a strong inhibitor or mineralization. Reduced levels were found both in murine PXE models and PXE patients. It is currently unknown, if serum level of PPi could be used as a

biomarker of disease severity or activity. We examined if plasma PPi level correlates with the extent of mid-dermal calcium deposition assessed by TPEF.

II. Experiment: To carry out spectral decomposition on *ex vivo* NLM images of PXE skin sections

4. Is Phloxine B staining suitable to create optical contrast between TPEF signal of elastin and calcification of deparaffinized PXE skin sections?

In our first experiment, TPEF signal of PXE skin was captured with one single bandpass filter. Later, we realized, that fluorescence that arises from the elastic fibres and calcium deposits cannot be spectrally separated. In this second experiment our aim was to identify the effect of an artificial low concentration Phloxine B staining, to create optical contrast between these tissue components.

5. Is it possible to spectrally separate collagen, elastin and calcification with mathematical algorithms, when three different bandpass filters are used to capture TPEF and SHG signals to create concentration maps of these tissue components and multicolor images of PXE sections?

We developed an inverse matrix based mathematical algorithm to spectrally separate TPEF and SHG signal of elastin, calcification and collagen. Our aim was to create

three-colored high-resolution images reflecting the actual chemical composition of these molecules in *ex vivo* PXE skin sections.

Methods

I. *Ex vivo* TPEF and SHG imaging in PXE

1.

50 μm thick unstained skin sections orthogonal to the skin surface were prepared from skin biopsy of 5 healthy individuals and 5 confirmed PXE patients. *Ex vivo* TPEF and SHG imaging was performed using a Ti-sapphire femtosecond pulse laser tuned to 800 nm central excitation wavelength. A commercial laser scanning two-photon microscope system was used to capture images. TPEF signal was collected with a 525/50 nm emission filter, SHG signal was separated with a 405/20 nm bandpass filter. Multiple TPEF and SHG images were captured from the same plane with 420x420 μm^2 individual field of view (FOV), which were assembled into 2-dimensional (2D) two-channel mosaic images with ImageJ image analysis software (NIH, Bethesda, USA). Parallelly to this experiment, histological analysis was performed. Besides conventional hematoxylin eosin staining, van Gieson, Weigert's elastic and von Kossa staining was carried out, which were used to validate nonlinear optical imaging.

2.

Raw TPEF images were used to analyse calcification and elastin. The number and length of the elastic fibres was manually counted and outlined using the ImageJ software. Calcium deposits were also outlined manually, and their relative surface area was calculated with ImageJ. Raw SHG images were used to measure length and width of the collagen fibres with the Curve Align and CT-FIRE softwares.

3.

Blood samples were collected from the five healthy controls and five PXE patients to measure plasma PPi levels. Plasma PPi content was compared between healthy individuals. PPi level of PXE patients were correlated with the measured relative surface area of calcification in their skin.

II. Spectral decomposition on *ex vivo* NLM images of PXE skin sections

4.

Skin sections from five PXE patients were included. One section was prepared from fresh-frozen PXE sample (PXE cryosection). The other four samples were prepared from formalin-fixed, and paraffin-embedded tissue blocks. After deparaffinization, these sections were stained with low concentration Phloxine B solution. NLM imaging was carried out with the same Ti:sapphire laser, however, an

additional 590/45 nm (orange) bandpass filter was used to capture TPEF signal. A gain calibration curve was determined for the comparison of the optical signal levels.

5.

For spectral decomposition of overlapping TPEF and SHG signals, an inverse matrix-based algorithm was developed. Matrix coefficients were determined in 132×132 μm^2 reference regions with the highest SHG (collagen), TPEFcyan (elastin) and TPEForange (calcium deposits) signal intensities, respectively. From the measured optical signal intensities and matrix coefficients, an inverse matrix was defined. This was used to deduce relative concentrations of tissue components at each pixel of the three channels in order to create concentration maps using the ImageJ software. Spectrally decomposed concentration maps of the three channels have been merged and assembled into three-color mosaic images.

Results

I. *Ex vivo* TPEF and SHG imaging in PXE

1. Similarly to the histological skin samples, the epidermis, papillary dermis and deep dermis was unaffected in all PXE patients. In healthy patients, we saw intact elastic fiber network in the TPEF channel, and interwoven collagen bundles in the SHG channel in the mid-dermis. On the contrary, mid-dermis of PXE patients

consisted of oval-shaped or confluent widespread calcified areas, surrounded by mineralized, widened, fragmented elastic fibers in the green TPEF channel. Calcification was present in the mid-dermis of all PXE patients. Conversely, we did not detect any calcification in controls. In the SHG channel of PXE patients we saw irregularly shaped collagen fibers.

2. Number of elastic fibers in the mid-dermis in PXE patients was found to be significant increased in PXE patients compared to controls. Elastic fiber length was significantly lower in PXE than in healthy subjects. Although calcification was present in the mid-dermis of every PXE patient, we found a considerable variation in the outlined relative surface area of calcium deposits between PXE patients. Analyzation of collagen fiber lenght and width with the CT-FIRE software resulted in significantly shorter collagen fiber length and decreased fiber width in PXE patients compared to controls, althouth the absolute difference in these values was not considerable.

3. Plasma PPi levels were significantly lower in PXE compared to healthy individuals. We found no correlation between relative surface area of calcification and plasma PPi levels.

II. Spectral decomposition on *ex vivo* NLM images of PXE skin sections

4. We experienced that emission spectra of elastin and calcium deposits can not be separated in a four color (magenta/cyan/green/orange) vectorial space in frozen PXE sections. However, the application of low concentration Phloxine B solution during the deparaffinization process evoked beneficial spectroscopic changes. TPEF signal of elastin remained prominent in the cyan channel, whereas calcification exhibited an increased TPEF signal captured with the orange bandpass filter. As a consequence, elastic fibers and calcium deposits could be optically distinguished based on their TPEF spectra.

5. Despite the considerable diversion of elastin and calcification color vector in case of Phloxine B stained sections, these two tissue components still emitted wide TPEF spectra and contributed with relatively high coefficients to cyan and orange channels, respectively. Spectral decomposition using the inverse matrices enabled us to separate optical signals originating from the three tissue components. Crosstalk of overlapping emission spectra was substantially decreased. In the output images, colors refer to the actual chemical concentration of the given tissue components. Calcified areas became clearly delineated. Fragmented elastic fibers could be distinguished from calcified elastic fibres.

Conclusions

I. Experiment: application of *ex vivo* TPEF and SHG techniques for the examination PXE-affected skin and its differentiation from healthy skin

1. Is *ex vivo* NLM suitable to visualize histopathological alterations of elastin and collagen, and reveal calcium deposits in PXE skin sections orthogonal to the skin surface with TPEF and SHG techniques?

To the best of our knowledge, we were the first in the literature, who successfully applied nonlinear optical microscopy methods in PXE patients to identify all histopathological skin alterations, including calcification in skin sections orthogonal to the skin surface. TPEF was able to visualize not just fragmentation of the elastic fibers, but calcium deposits and mineralized elastic fibers were also visible. These detrimental changes were absent from the skin of healthy individuals, as expected. As we applied one bandpass filter to capture TPEF signal, elastin from calcium deposits were distinguished based on their morphology. SHG revealed clear morphology of collagen bundles. Our results were compared and validated by conventional histopathological stains, such as H&E, WE, VG and VK stains. Thus we concluded, that nonlinear optical microscopy modalities are suitable to detect connective tissue alterations and calcification present in

PXE-affected skin in a label-free manner with high spatial resolution.

2. Do quantitative parameters of elastin and collagen fibres differ between healthy controls and PXE patients?

We introduced numerical methods for quantitative and semi-quantitative analysis of PXE specific mid-dermal alterations. We found signicantly shorter and higher number of elastic fibres in PXE patients. Although calcification was absent from healthy skin, its relative surface area varied considerably among PXE patients. Collagen fibre length and width was significantly lower in PXE patients, although the difference in these parameters was clinically not considerable.

3. How variable is the extent of calcification in the mid-dermis? Does this correlate with a possible soluble biomarker, PPi?

Plasma concentration of PPi, a potent inhibitor of mineralization was significantly reduced in PXE patients compared to healthy individuals. In our cohort, PPi did not correlate with the extent of mid-dermal calcium deposition.

Based on our experiments, *ex vivo* nonlinear optical microscopy was validated as a tool to identify characteristic histopathological alterations in PXE-

affected skin in a label-free manner. This method might be used in the future in the diagnosis of PXE or for the assessment of skin status of PXE patients.

II. Experiment: High-chemical contrast imaging and spectral decomposition on *ex vivo* TPEF and SHG images of PXE skin sections

4. Is Phloxine B staining suitable to create optical contrast between TPEF signal of elastin and calcification of deparaffinized PXE skin sections?

The application of low concentration Phloxine B staining during deparaffinization created optical contrast between the emitted TPEF signal of elastin and calcification. This became visible, when the emitted TPEF and SHG signals of collagen, elastin and calfication were captured by three different bandpass filters.

5. Is it possible to spectrally separate collagen, elastin and calcification with mathematical algorithms, when three different bandpass filters are used to capture TPEF and SHG signals to create concentration maps of these tissue components and multicolor images of PXE sections?

Determination of contribution of collagen, elastin and calcification to the TPEF and SHG signals intensities at each detection channel enabled spectral unmixing of their overlapping emission spectra. With the applied image

procession algorithm, chemical selectivity considerably increased and three-color mosaic images could be created, where colors reflected actual concentration of these dermal tissue components.

Our results have considerable benefits compared to conventional histopathology since spectrally unmixed NLM images can be directly used to carry out quantitative analysis on the extent of mid-dermal calcium deposition or to examine the pattern of calcification. Furthermore, fine alterations to elastic fibers could be better identified due to higher chemical selectivity and higher spatial resolution of NLM. In the future, three-channel imaging and spectral unmixing of *ex vivo* deparaffinized PXE skin samples may be used in the future for quantitative analysis during the diagnostics or monitoring of patients. This method could also be utilized in case of other diseases with ectopic calcification.

Publications

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