Assessing the application possibilities in dermatologyand potential risks of pulsed lasers used in nonlinear microscopy

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Introduction

In the last few decades nonlinear optical techniques have undergone a rapid development, numerous methods have been made into commercially available devices that are able to visualize the skin *in vivo*. Laser sources tuned in the near infrared spectral range penetrate into the deeper layers of the skin, where the excitation of endogenous chromophores allows a safe imaging with high spatial and temporal resolution. By using the different nonlinear optical imaging techniques, such as twophoton excitation fluorescence (TPEF), second harmonic generation (SHG) or coherent anti-Stokes Raman scattering (CARS) the whole cross section of the skin can be visualized. The TPEF technique is able to detect the keratin, NADH, melanin or elastin, and with using the SHG several non-symmetric molecules, such as collagen. The CARS technique uses laser lights with different wavelengths focused on one spot. This results in an optical signal specific to a given molecule or tissue, thus intracellular lipids and subcutaneous adipocytes are easily identified.

Our research group collaborates since 2009 with a team of physicists to develop a compact, cost effective laser scanning microscope that can be used in the dermatologist's office. Our measurements were performed on a conventional Zeiss twophoton microscope, and in our work we examined skin structure alterations that often occur in daily clinical practice and

affect many patients. In our present work we examined the application possibilities of the nonlinear optical techniques, and our results provided basic technical information, which are necessary in further construction and designing the handheld device.

Aims of the study

1. Monitoring the penetration of nanomedicines through the skin by using *in vivo* nonlinear microscopy

The epidermal bone marrow-derived dentritic cells, so-called Langerhans cells function as antigen presenting cells in the skin. Following the uptake of antigen the cell migrate to lymph nodes via lymphatic vessels, then they presenting antigens for T-cells. Transcutaneous immunization refers to the topical application of antigens into the epidermis and following the uptake and presentation it enables efficient immunization of whole body against specific antigens. In our study we examined the penetration of Alexa546 labeled and label-free DermaVir nanoparticles in Langerin-EGFP *knock in* mice using *in vivo* CARS methods and the *FiberScope* imaging device.

2. The effect of metabolic syndrome to the skin - examination of dermal collagen and subcutaneous adipocytes of leptin deficient obese mice by using *in vivo* SHG and *ex vivo* CARS imaging techniques

The prevalence of obesity and type 2 diabetes show a steady increase worldwide, they are responsible for the development of several chronic diseases. In addition, the incidence of metabolic syndrome-related skin disorders is fairly high in the Western population. In obesity, adipocytes grow in size and can cause the degradation of dermal collagen network via production of inflammatory cytokines, free fatty acids and free radicals. The purpose of our work was to identify the main features and structural changes of diabetic murine skin by *in vivo* SHG methods. At the end of the study the size of adipocytes was also examined by using *ex vivo* CARS technique.

3. The effect of metabolic syndrome to skin - examination of dermal collagen and subcutaneous adipocytes induced by various diets using *in vivo* SHG and *ex vivo* CARS imaging techniques

For a more accurate modeling of human obesity and unhealthy diet, we induced obesity by high fat and/or fructose diets on mice with wild-type genetic background. In this long term experiment we examined the main structural alterations of dermal collagen and

subcutaneous adipocytes by using *in vivo* SHG and *ex vivo* CARS methods, similarly to our previous study.

4. Assessment of possible health risks of titan-sapphire and ytterbium laser sources

High-intensity pulsed laser light sources used in nonlinear microscopes can damage the samples when settings are incorrect. In this part we found important to examine the possible photochemical and thermal damage mechanism of our TiS solid state and Yb fiber laser sources, due to the contradictory results in various publications. During the excitation of endogenous chromophores – similarly to UVB radiation – cyclobutane pyrimidine dimers are being formed in the DNA, which may lead to mutations. Furthermore, there is a possibility of thermal tissue damages caused by the laser sources tuned in the infrared spectral range. Next to safety second priority in clinical diagnosis is the capturing of good quality, high resolution images. In order to achieve them we have to define the optimal parameters, such as wavelength, laser power and pixel dwell time. In this work our aim was to identify the various damage mechanisms and to specify the proper laser parameters that are necessary for safe, good quality imaging.

Methods

1. Monitoring the penetration of nanomedicines through the skin by using *in vivo* nonlinear microscopy

In our experiment we used 10 weeks old Langerin-EGFP-DTR *knock in* mice. In these animals the epidermal Langerhans cells are labeled with green fluorescent protein (GFP). Mice were anesthetized with 1.2% Avertin solution (0.23 mL/10 g, Sigma–Aldrich, Hungary) intraperitoneally, then their ear were attached microscope slides. The interruption of stratum corneum was performed by using DermaPrep (Genetic Immunity, Hungary) medical sponge. In the labeled DermaVir nanoparticles the Alexa546 covalently binding to a polyethyleneimine-mannose (PEIm) nanoparticle which containing the plasmid DNA. To the ear skin surface ~ 2 µl DermaVir solution was dropped, allowed to dry and the penetration was measured after 1 h *in vivo*. Through the *in vivo* CARS measuring we detected the Alexa546-labeled DermaVir, then examinations with *FiberScope* device we used the label-free nanoparticles.

2. The effect of metabolic syndrome to skin - examination of dermal collagen and subcutaneous adipocytes of leptin deficient obese mice by using *in vivo* SHG and *ex vivo* CARS imaging techniques

The 8-week-old female mice deficient for leptin (B6.V-Lep ob/ob) (Charles River, Italy) were divided into two groups. There were two mice in each group, and they were kept on two kinds of diet for 30 weeks. The first ob/ob group received a normal diet *ad libitum*, while the second ob/ob group was kept on a calorie-restricted diet. We used C57BL/6J mice on normal diet as controls. The body weight of each mouse was measured weekly. *In vivo* SHG imaging technique of the collagen was performed four times on week 8, 16, 24 and 30. The Ti:S laser source was tuned to 795 nm and the mean power was 27-30 mW. In order to achieve detailed, high quality images, 1024 x 1024 resolution, 12,61 µsec pixel dwell time and 2 averaging was used. We captured five image series form each mouse up to 60 µm depth with 20x power magnification. To assess the size of adipocytes we used *ex vivo* CARS technique. In this case 4 mm full-thickness punch biopsies were taken from dorsal skinand placed on a microscope slide upside down. Three z-stack measurements were performed on each mouse. At the end of the experiment, a histological analysis was performed on each sample. Besides conventional hematoxylin eosin staining, van Gieson staining was also performed. The dermal thickness and size of adipocytes were evaluated using the Digimizer Image Analysis Software (Ostend, Belgium). The value of SHG intensity was calculated by WCIF ImageJ image analysis software. Because of the low number of mice in the three groups

accurate statistical probes could not be used. Instead SHG intensity results were normalized referring to the control group and we compared the mean values of various groups to controls. Regarding the dermal thickness and size of adipocytes we also compared the mean result to each other.

3. The effect of metabolic syndrome to skin - examination of dermal collagen and subcutaneous adipocytes induced by various diets using *in vivo* SHG and *ex vivo* CARS imaging techniques

In this 32 week long experiment we followed the effect of various diets to dermal collagen and subcutaneous adipocytes. We investigated 8 week old female C57BL/6 mice, 5 mice per groups. Mice were divided in to the following groups:

- High fat group (HFat) – received 30% fat containing chow for induction of obesity
- High fructose group (HFru)–received 20% fructose enriched drinking water for induction of collagen glycation processes
- High fat high fructose group (HFHF) – received high fat and high fructose diet for induction of obesity and collagen glycation
- Control group – standard chow and water

To follow the structural changes of dermal collagen we used the SHG method four times *in vivo* during our experiment at weeks 8,16,24 and 32. The main settings were not changed, however in order to accelerate imaging the resolution and pixel dwell time were decreased. The value of SHG intensity was calculated by WCIF ImageJ image analysis software. The mean SHG intensity of given groups were compared with one-way ANOVA and Student's T-test. The dermal thickness and sizes of adipocytes were evaluated on the van Gieson stained sections using Digimizer Image Analysis Software (Ostend, Belgium).

4. Assessing the possible risks of titanium-sapphire and ytterbium laser sources

During our experiments we investigated 8 week old C57BL/6 mice. Mice were anesthetized with 1.2% Avertin solution (0.23 mL/10 g, Sigma–Aldrich, Hungary) intraperitoneally, thereafter the fur was completely removed from the dorsal skin by shaving. We marked a 5 mm x 10 mm rectangular area on the dorsal skin, which were irradiated in horizontal directions. The irradiation method was performed by a Zeiss two photon microscope (Axio Examiner LSM 7 Carl Zeiss, Germany), with a tunable, femtosecond pulse Ti:S laser source. The main settings of irradiation methods are reviewed in Table 1.

	Wavelenght	Power	Resolution	Pixel Dwell Time	Averaging	Z-stack/ Interval	Objective
1.	Ti:S 750 nm	~ 60 mW	512 x 512	1,58 µsec	16	30 µm/ 3 µm	20x W
2.	Ti:S 750 nm	~ 25 mW	1024 x 1024	6,3 µsec	2	30 µm/ 3 µm	20x W
3.	Ti:S 800 nm	~ 25 mW	1024 x 1024	6,3 µsec	2	30 µm/ 3 µm	20x W
4.	Ti:S 1030 nm	~ 25 mW	1024 x 1024	6,3 µsec	2	30 µm/ 3 µm	20x W
	pozitív kontroll – UVB 312 nm – 1 J/ cm^2 – 20 perc						

Similarly to previous irradiation procedures we also assessed the potential risks of Yb fiber laser source. Here we used the *FiberScope*-equivalent settings: 20x water immersion objective, 303 µm x 303 µm image areas, 512 x 512 resolutions, 25 µsec pixel dwell time, 6 mW average power and 1030 nm wavelength. After irradiation dorsal skin was removedand fixed in formalin, embedded in paraffin and sectioned vertically, then we carried out the immunofluorescence labeling. We also used UVB irradiated murine skin sections as positive controls.

Results

1. The results of investigation the penetration of nanomedicines

During the CARS measurements we observed that the CARS signal from intercellular lipids and other lipid-containing cell components overlap the signal of PEIm components of DermaVir. This problem significantly affected the selectivity of our measurements. In the further examination by *FiberScope*, similarly to our previous work, we detected the uptake of Alexa546-labeled nanoparticles by Langerhans cells in the DermaPrep treated skin after one hour, and marked immune cells in the "red" channel of our device.

2. Effect of obesity to the skin – investigation of leptin deficient ob/ob mice

At week 4 the leptin deficient ob/ob mice showed a rapid increase in body weight. The calorie restricted leptin deficient group had a comparable body weight to controls. At week 30 the weight gain in the ob/ob-ND group (mean 63,35 g ± 4,31 g) was three times higher, than in control (mean 26,63 g ± 4,12 g) and ob/ob-CRD (mean 30,33 g ± 1,39 g) groups. During the first three measuring sessions, we could not observe significant differences in the SHG intensities and collagen morphology among the groups. At the final evaluation, at week 30, the collagen morphology in the ob/ob-CRD

group was normal and fiber-rich, just as in the in control animals. In contrast to that in the ob/ob—ND group a markedly reduced dermal collagen content and decreased SHG intensity were recorded. The *ex vivo* CARS imaging method was performed once at the end of the study. According to our expectations the subcutaneous adipocytes in the ob/ob-ND group were notably larger compared to the control group. Histological evaluation revealed 2.5 times greater adipocyte sizes in the ob/ob – ND group (3678,06 μm^2 ± 984,95 μm^2) than in control mice (1429,1 μm^2 ± 316,25 μm^2). Along with the enlarged adipocytes in ob/ob-ND group, the dermal thickness decreased greatly (115,2 μm ± 27,35 μm) compared to control animals 210,42 μm ± 8,4 μm).

3. Effect of obesity to the skin – effects of various diets.

Mice in HFat group had significantly higher body weight from week 12 on ($p < 0.05$) than controls, while the weight gain in HFHF group was only moderate. The body weight of animals in the high fructose group was relatively constant during the 32 weeks; the increase was similar to control mice. The degree of SHG intensity significantly decreased in HFat and HFHF groups compared to controls at second measuring session. Furthermore in HFru group the SHG intensity was significantly lower at week 24 and 32 than in control animals. Certain degradation of dermal collagen was

mainly observed in HFat group parallel to the degree of obesity. The structural changes of collagen in HFru group underwent a moderate alteration, however at week 32 the fragmentation of collagen fibers was clearly visible. On *ex vivo* CARS images we could see that fat cells in HFat- and HFHF-induced obese mice were much larger than in the control and HFru groups. Histological evaluation of dermal thickness showed a significant decrease in HFat (120,15 μm ± 14,56 μm) and HFru-diet-fed (141,23 μm ± 12,65 μm) mice compared to controls (155,58 μm ± 12,36 μm) ($p < 0,05$). At the same time the size of adipocytes was significantly larger in mice of HFat (233,15 μm ± 22,84 μm) and HFHF (219,63 μm ± 21,84 μm) groups than control (145,13 μm ± 14,93 μm) and HFru-diet-fed (154,05 μm ± 21,19 μm) animals.

4. The results of the risk evaluation of laser sources

Skin samples that have been exposed at 750 nm to a relatively large laser power (60 mW) showed a few CPD positive cells with very weak fluorescent intensity compared to positive controls. Other skin sections after an exposure at 800 nm and 1030 nm wavelengths to similar power levels were CPD negative. Similarly the sections which were exposed to Yb fiber source were also CPD negative. Thermal damage could be achieved only when multiple power levels were used (100-130 mW), however, these power levels are rarely required for *in vivo* imaging (25-50 mW).

Conclusions

1. By using *in vivo* CARS technique we assumed that the label-free PEIm component of DermaVir solution is excitable with high efficacy, thus the nanoparticles become identifiable. However during the CARS measurements we observed that the CARS signal from intercellular lipids and other lipid-containing cell components overlapped the signal of PEIm components of DermaVir, therefore the nanoparticle-derived CARS signal was not distinguishable from other cell component-derived signals. In subsequent examination by *FiberScope*, we were able to detect the uptake of Alexa546-labeled nanoparticles by Langerhans cells in the DermaPrep treated skin after one hour, and the nanomedicines marked immune cells. With this experiment we demonstrate the efficient applicability of *FiberScope* device in pharmacological investigations.

2. Extreme obesity in leptin deficient mice induced notable dermal collagen degradation, decrease of dermal thickness and adipocyte enlargement. A restriction in the calorie uptake could successfully prevent these processes. In our work we were able to follow the main structural alterations in the skin of genetically obese mice, using *in vivo* SHG and *ex vivo* CARS nonlinear optical methods. Our observations were in accordance with previous results, which are already explained by molecular biological experiments.

3. The high fat and/or high fructose diets also induced the decrease of dermal collagen and fragmentations of fibers. This was mainly seen in the HFat group, where a significant body weight gain was also observed. The 20% fructose enriched water did not cause remarkable obesity, however the degradation of dermal collagen was clearly visible. The dermal thickness did not show significant decreasing in the HFHF group, but the structural changes were also notable. In our study we demonstrated again that the nonlinear optical methods are able to follow the structural changes of connective tissue both *in vivo* and *ex vivo*. Additionally, our results prove and highlight the importance of body weight control and healthy nutrition.

4. We also demonstrated that the high-intensity, pulsed laser light sources used in nonlinear microscopes with proper settings allow safe imaging and the techniques are capable of *in vivo* investigations. Our results were in line with previous publications, which also confirmed, that the damages, which possibly can occur during *in vivo* nonlinear imaging, are negligible.

Publications

Publications related to the thesis

Kolonics A, CsiszovszkiZs, Tőke ER, Lőrincz O, **Haluszka D**, Szipőcs R. (2014)
In vivo study of targeted nanomedicine delivery into Langerhans cells by multiphoton laser scanning microscopy. Exp Dermatol, 23(8): 596-605

Haluszka D, Lőrincz K, Csákányi A, Vass L, Krolopp Á, Kolonics A, Szipőcs R, Kárpáti S, Wikonkál N. (2015) Nemlineáris mikroszkópia alapjai és alkalmazási lehetőségei a dermatológiában. Bőrgyógy Ven Sz, 91(1): 34-39

Haluszka D, Lőrincz K, Molnár G, Tamás G, Kolonics A, Szipőcs R, Kárpáti S, Wikonkál NM. (2015)
In vivo second-harmonic generation and ex vivo coherent anti-Stokes Raman scattering microscopy to study the effect of obesity to fibroblast cell function using an Yb-fiber laser-based CARS extension unit. Microsc Res Tech,78(9):823-30

Publications regardless the thesis

Bognár P, Németh I, Mayer B, **Haluszka D**, Wikonkál NM, Ostorházi E, John S, Paulsson M, Smyth N, Pásztói M, Buzás EI, Szipőcs R, Kolonics A, Temesvári E, Kárpáti S. (2013)
Reduced inflammatory threshold indicates skin barrier defect in transglutaminase 3 knockout mice. J Invest Dermatol, 134(1): 105-11

Lőrincz K, Bánvölgyi A, **Haluszka D**, Keszeg A, Márton D, Kuroli E, Szipőcs R, Scharffetter-Kochanek K, Wlaschek M, Kárpáti S, Wikonkál N. (2015)
Hosszútávú PUVA kezelés bőr öregítő hatásának vizsgálata egereken. Bőrgyógy Ven Sz 91(1): 18-22