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# Clinical study on the effects of a cosmetic product on dermal extracellular matrix components using a high-resolution multiphoton tomograph

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**Background/purpose:** The aim of this study was to demonstrate the effects of selected plant extracts in a cosmetic cream on the dermal network components after a 3-month treatment using an *in vivo* multiphoton tomographic device.
**Methods:** Twenty-four Caucasian women aged between 45 and 65 applied randomly a cosmetic emulsion B containing active ingredients (soy and jasmine) twice a day on one arm and its vehicle A (without active ingredients) on the other arm during 3 months. Measurements were performed on the internal side of the forearm before starting the treatment (T0), after 4 week (T4) and 12 weeks (T12) treatment. Measurements consisted in a multi-layers acquisitions using a multiphoton tomograph with subcellular resolution. Optical sections (about 6 μm thick) were recorded from 0 to about 200 μm using two different wavelengths: 760 and 820 nm. To compare the series of images and obtain an objective quantification of the signal of second harmonic generation (SHG) and autofluorescence, the method used consisted in taking the integrated brightness of an image (same rectangular area for all images) as a measure of the signal. Following this step a ratio between brightness of images from the area treated with cream A or B and brightness of untreated area was calculated and used as an assessment of treatment efficacy. The parameter used for statistical analysis (variance analysis) is the difference before and after 12 weeks of treatment by either cream A or B of the signal ratios calculated in the upper dermis (118–130 μm) and those from a deeper region of the upper dermis (165–178 μm).
**Results:** Signals (autofluorescence+SHG) of extracellular matrix do not change significantly with time (weeks 0, 4 and 12) when cream A (vehicle with no active ingredient) is applied. Treatment with cream B results in an enhancement in the signal level of extracellular matrix at week 12. The comparison of signals, in both areas (118–130 μm and 160–178 μm), show an higher increase in the deeper region than in the more superficial one for product B while we do not notice any change with product A.
**Conclusion:** The multiphoton tomograph provided excellent high-resolution images, which describe clearly the different skin layers, single cells and extracellular matrix components in all the 24 volunteers. Statistic analyses reveal a real effect for product B with selected plant extracts, known to increase collagen synthesis. Changes observed are characteristics of modifications in dermal collagen and elastin content. To our knowledge, it is the first time that it was possible to demonstrate *in vivo* the effect of a cosmetic product on the superficial dermal layer, in a non-invasive and non-destructive process, i.e. without cutting the skin.

**Key words:** Multiphoton Spectroscopy – Multiphoton tomography – extracellular matrix – second harmonic generation – dermal structures


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MULTIPHOTON FLUOROPHORE excitation using near-infrared (NIR) femtosecond laser pulses and high numerical aperture (NA) objectives can induce a weak autofluorescence based on naturally occurring endogenous fluorescent biomolecules such as flavins, reduced nicotinamide adenine dinucleotide phosphate [NAD(P)H] coenzymes and metal-free porphyrins as well as components of lipofuscin, collagen, elastin and keratin. Autofluorescence (AF) and second harmonic generation (SHG) can be used to perform optical imaging of the structural components of tissues (1–9).

NIR intensities as high as a gigawatt per square centimeter can be applied without any damage to tissues and fluorophores and deep tissue imaging can be obtained. Multiphoton excitation occurs only in a tiny intra-tissue focal volume of about one tenth of femtoliter ($10^{-15}$ L).

Multiphoton microscopes are not suitable as laboratory devices for *in vivo* imaging in humans. A multiphoton tomograph has been especially developed for high-resolution imaging of the human skin (10, 11).

In contrast to the standard technique for determining the histopathology of mechanically removed tissue, non-invasive three-dimensional (3D) optical diagnosis has the advantage of (1) providing a painless diagnosis method without tissue removal, (2) rapid access to information, (3) examination under natural physiological (*in vivo*) conditions and (4) the possibility of long-term studies on the same tissue area including *in vivo* drug screening.

The aim of this study was to demonstrate the potential of this technique to assess the effects of selected plant extracts in a cosmetic cream on collected AF and SHG signals by performing and analyzing images of the epidermal and dermal structures.

Here, we report a novel application of the femtosecond laser scanning system to non-invasive high-resolution multiphoton tomography of *in vivo* human skin with the capability to study the effects of a cosmetic product with submicron spatial resolution.

## Material and Methods

The multiphoton tomograph DermaInspect device, as shown in Figs 1 and 2, consists of a compact, solid-state, turnkey, modelocked 80 MHz titanium sapphire laser MaiTai (Spectra Physics, Mountain View, USA) with a tuning range of 750–850 nm coupled into a scanning module with a fast x,y galvoscanner, piezodriven $\times 40$ focusing optics with high NA 1.3 (oil) and 200 µm working distance. A fast PMT detector module and a control unit including JenLab Image software (JenLab GmbH, Jena, Germany) for image processing were used.

*Fig. 1. Multiphoton tomograph DermaInspect device.*

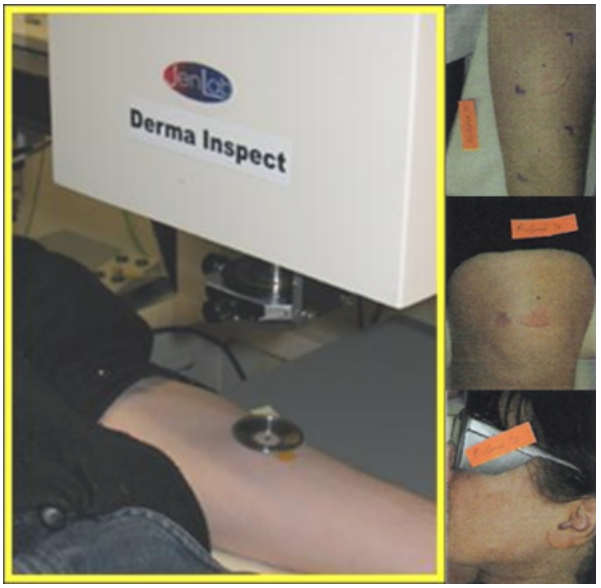


*Fig. 2. Optical sections have been performed at different tissue depths ranging from 0 µm (surface) down to about 200 µm on the inner side of the forearm, on the upper the knee and on the face.*

Investigations on the human skin using Multiphoton tomography with a subcellular resolution were performed in 24 women aged between 45 and 65 years who applied a test cosmetic cream twice a day. The study was carried out after signed informed consent was given by all participants and was approved by the local ethic committee. Measurements were performed on the internal side of the forearm before starting the treatment (T0) and after a 4-week (T4) and a 12-week (T12) treatment. Additional measurements were performed on the face and on the upper level of the knee (Fig. 3, untreated areas).

Multiphoton tomography was performed at the Clinic of Dermatology, Friedrich Schiller Universität (FSU), Jena, between June 2005 and November 2005.

Optical sections (5000 in total) were recorded on the internal side of the forearm (all volunteers), on the upper level of the knee and on the face (in two volunteers) as shown in Fig. 3 at different tissue depths ranging from 0 µm (surface) down to about 200 µm using two different laser wavelengths: 760 and 820 nm. The acquisition of one section (5.9 µ) took 7.4 s.

Measurements were carried out at T0, T4 and T12 on (i) a defined untreated site as a control, (ii) a defined site treated with test cream A

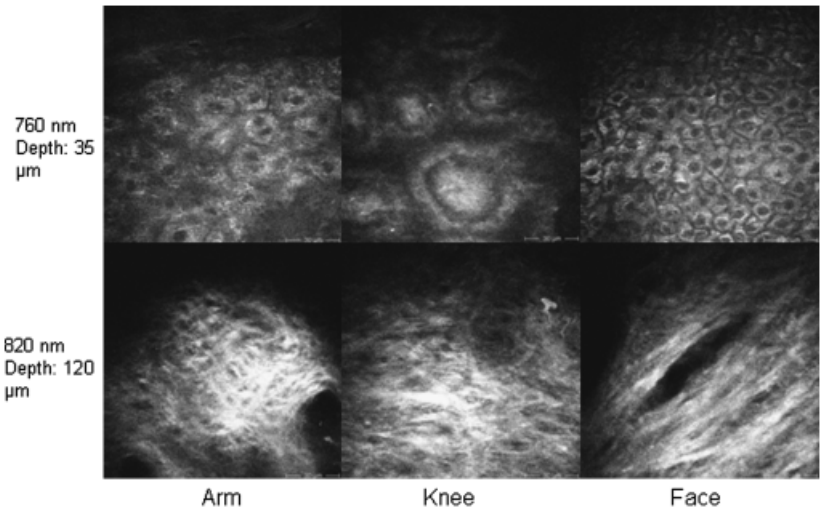


*Fig. 3. Examples of high-quality images obtained at 760 and 820 nm laser wavelength excitations on forearm knee or face skin tissue.*

(cosmetic emulsion without active compounds, the vehicle) and (iii) a defined site treated with cream B (the vehicle with soy and jasmine extracts). The sites were randomly and blindly chosen. The active ingredient is a complex of three active components from two selected extracts (soy and jasmine) known to contribute to collagen and decorin synthesis.

We used a laser wavelength of 760 nm to obtain a two-photon luminescence (fluorescence, SHG) signal of all the cells and of extracellular matrix (ECM) components with a submicron lateral resolution and a 1–3 µm axial resolution. One can observe that the different characteristic structures of the *stratum corneum*, including hexagonal-shaped cells, are found at a depth from 0 to 15 µm. When focusing deeper (Fig. 3), the signal within the *stratum granulosum* and within the *stratum spinosum* is determined by mitochondrial NAD(P)H fluorescence. The fluorescence is brighter and influenced by melanin emission *from* the *stratum basale*.

Interestingly, the ECM elements could be imaged with a high signal-to-noise ratio in all the volunteers up to a depth determined by the working distance of the focusing optics of 200 µm. The mean laser power was varied from 12 to 49 mW to record efficient AF signals depending on the intra-tissue focal spot.

The second measurement series was performed at a laser wavelength of 820 nm. At this wavelength, the signal is mainly produced by collagen-related SHG and elastin-related AF. The SHG signals produced by collagen consist of radiation at half the incident laser wavelength. Examples of high-quality images obtained are presented in Fig. 3. Whereas at 760 nm laser wavelength excitation, one can observe the stratum spinosum of the epidermis at a depth of 35 µm, deeper sections of the tissue can be reached when using an 820 nm laser wavelength: images of elastin and collagen in the dermis are thus obtained from the forearm, the knee or the face.

## Statistical Processing

A statistical approach of product effects with time was carried out. The objective was to compare the amount of recorded luminescence signal in the dermis at an 820 nm laser wavelength in cream A- (cosmetic emulsion without active compounds, the vehicle) vs. cream B (the vehicle with soy and jasmine extracts)-treated skin areas at T0, T4 and T12, at the same depth and the same excitation laser power. To compare the series of images and to obtain an objective quantification of the signal of SHG and AF, the method used consisted of taking the integrated brightness of an image (same rectangular area for all images) as a measure of the signal. The integrated brightness was defined as the sum of pixels weighted over the brightness values (or gray values) of the pixel $\sum_{i=0}^{255} i^* N_i$; where $N_i$ is the number of pixels with gray value $i$.

Thus, the more intense the SHG and AF signals, the higher the integrated brightness, and, when there is no signal, the image is dark, i.e., all pixels have 0 grayness and integrated brightness is also 0.

The normalization of brightness was carried out for tomography images in the dermis at a fixed depth beginning at 88.5 μm from the surface just under the *stratum basale* of volunteers (average value over several volunteers deduced from the images) up to 180 μm. As a measurement of the effect of the treatment, the ratio of the brightness of images from the area treated with cream A or B to that of images from the untreated area was used. The following notations were used:

- $R$, $A$, $B$ – signal from untreated (control) skin, product A-treated skin and product B-treated skin, respectively,
- *week*0, *week*4, *week*12 – treatment period,
- *volunteer.N* – volunteer number N,
- *depth* – skin depths considered: 88–178 μm,
- $\bar{R}_{\sum weeks}$, average value of the control skin signals for the three measurement periods: *week*0, *week*4, *week*12.

Between 88 and 118 μ, due to the automation of the quantification process, it is possible to take into account some epidermis information like flattening of the dermal–epidermal junction. Hence, to restrict our conclusion on dermis modification, we chose to carry out statistical analysis between 118 and 178 μ.

A one-way variance analysis ($\alpha = 0.05$) was used to make an inference on the statistical significance of difference before and after T12 weeks of treatment by cream A and cream B signal ratios calculated in the upper dermis L2 (118–130 μ) and those from a deeper region of the upper dermis L1 (165–178 μ).

## Results

The ratio of the signal for cream A-treated skin is defined as

$$\frac{A_{week12,\,depth,\,volunteer}}{R_{depth,volunteer,\sum weeks}} \quad (1)$$

and for cream B-treated skin as

$$\frac{B_{week12,\,depth,\,volunteer}}{R_{depth,volunteer,\sum weeks}} \quad (2)$$

The signal of untreated skin serves as a control.

To quantify the effects of the treatment averaged for all the volunteers, the ratios should be considered as in Eqs (3) and (4). The ratios of the AF and SHG signals and the significant difference after 3 months of product application are given in Table 1 for product A and product B.

$$\frac{A_{depth,week,\sum volunteers}}{R_{depth,week,\sum volunteers}} \quad (3)$$

and

$$\frac{B_{depth,week,\sum volunteers}}{R_{depth,week,\sum volunteers}} \quad (4)$$

The evolution of the ratios for product A and product B at T0, T4 and T12 weeks is summarized in Figs 4 and 5, respectively.

The bars on the left side of the curves show the value of variance – one value of variance per line. The error bars on the right side of the curves denote the experimental error.

The high level of variance is mainly due to movement artifacts of the *in vivo* measurements. In this respect, the results presented above reduce the set of data by excluding the signal values that

*TABLE 1. Summary of variance analysis results on the difference in the ratios of SHG and AF signals before and after 12 weeks of treatment showing a significant difference on the L1 region after a 3-month product application*

| Region of interest | Effect of active ingredients on dermal components* | *F* value** | *P* value*** |
|---|---|---|---|
| L2<br>118 to 130 μ | 0.29 | 1.07 | $3.10^{-1}$ |
| L1<br>160 to 178 μ | 0.63 | 14.61 | $10^{-6}$ |

*This parameter is the difference between the difference of ratios before (T0) and after treatment (T12weeks) for product A and product B.
**The *F* value is obtained from one-way variance analysis.
***$P$ value is significant when $< 5 \times 10^{-2}$.

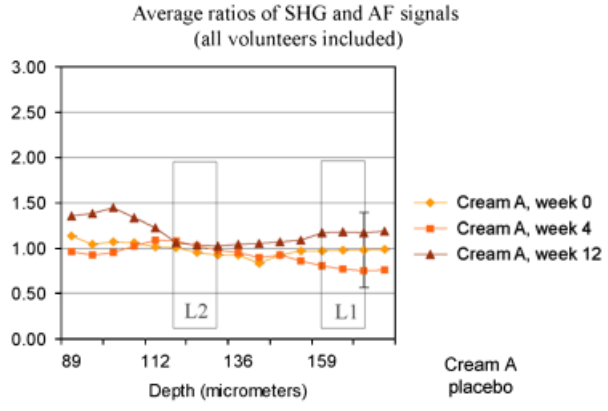


*Fig. 4. Changes in the ratios of second harmonic generation (SHG) and autofluorescence (AF) signals resulting from treatment with cream A. (ratios at T0, T4 and T12 weeks averaged for all the volunteers). The error bars on the right side of curves denote the experimental error.*

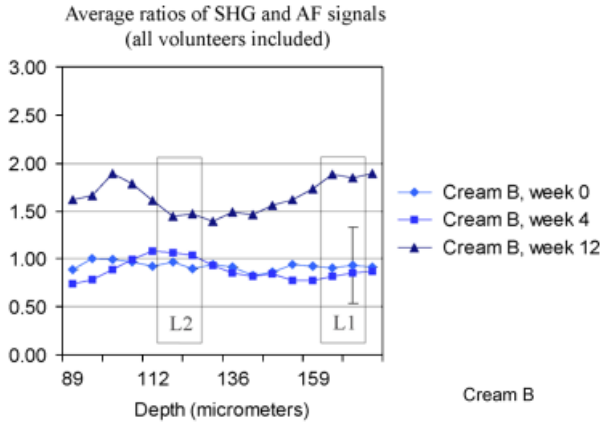


*Fig. 5. Changes in the ratios of second harmonic generation (SHG) and autofluorescence (AF) signals resulting from treatment with cream B. (ratios at T0, T4 and T12 weeks averaged for all the volunteers). The error bars on the right side of the curves denote the experimental error.*

deviate about threefold from the mean values (exclusion of 12%).

We notice that the signals (AF+SHG) of ECM do not change significantly with time (weeks 0, 4 and 12) where cream A (vehicle with no active compound) is applied. Treatment with cream B results in an enhancement in the signal level of ECM at week 12.

The results of the comparison of signals in L1 and L2 regions for both products A and B are summarized in Table 1. The parameter used is the difference between T0 and T12 weeks of the ratio for product A and the same difference for ratio for product B. This parameter represents the effect of active ingredients on dermal components.

## Discussion

To our knowledge, it is the first time that multiphotonic tomography is used to study *in vivo* the effects of cosmetic products on the skin structures. The results clearly demonstrate that the level of global signals detected in the dermis is higher after treatment with cream B, whereas no significant alteration is noticed following treatment with vehicle (cream A). This suggests that cream B may have induced changes in the structure and/or the composition of the components of dermal tissue.

Dermal collagen and elastin content are modified in aged skin as well as in pathological skin conditions: skin aging is associated with an impaired collagen and elastin fibrillar structure. In photoaging, for instance, this decrease is explained mainly by the UV-induced expression of matrix metalloproteases (MMP) that digest dermal collagen (12, 13). The second important hallmark of skin photoaging is the extensive degradation or the significant alteration of the elastic fiber network, which might also be caused by MMPs. The so-called elastosis, the deposit of truncated elastotic material, is thought to be caused by the direct impact of UV on the synthesis of elastic fibers. Here, data acquisitions have been carried out on the internal side of the forearm where photoaging is weak, in order to focus our conclusion only on chronological skin aging.

ECM modifications linked to aging have been shown to be prevalent in the superficial dermal layer (14, 15). Hence, we carried out our statistical analysis in the upper part of the dermal layer. Statistical analysis of data showed that, after 3-month applications of product B, the level of signal increases in the upper part of the dermal layer from 118 to 178 µm depth. Interestingly, this difference is significant from 165 to 178 µm, where measurement takes place in the upper dermis close to the region of transition between the papillary dermis and the reticular dermis.

An important parameter of skin aging is the dermal collagen content. Collagen I accounts for 70–90% of dermal ECM and its content decreases by about 1% per year. Recently, multiphoton laser tomography studies in the human dermis of 18 volunteers of different ages have been shown to well suited to characterize human dermis age (16). In our study, the AF and SHG signals were not recorded separately, but the fact that the global signal had increased after the 3-month treatment with cream B suggests that some changes have occurred in the composition of the collagen fibers and/or the elastin in terms of structure, proportion or density. Cream B is known to enhance collagen synthesis; hence, we suspect that this signal increase is mainly linked to collagen synthesis. To demonstrate this hypothesis, additional studies are in progress, taking into account the evolution of the DermaInspect system, which now allow to separately measure the SHG and AF signals (14). Hence, we will be able to quantitatively determine the ratio between SHG and AF signals and characterize its modification after cosmetic treatment.

## Conclusion

The DermaInspect device provided excellent high-resolution multiphoton images, which

show clearly the different skin layers, single cells and ECM components in all the 24 volunteers.

Interestingly, the signal depth could be enhanced down to about 200 µm and was limited due to the working distance of the focusing optics. Statistic analyses reveal a real effect for product B with selected plant extracts known to increase collagen synthesis. The changes observed are characteristics of modifications in dermal collagen and elastin content. Further studies are in progress to quantitatively evaluate the alterations in skin composition over time and the impact of test plant extracts on collagen and elastin fibers separately.

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## References

1. Denk W, Strickler JH, Webb WW. Two-photon laser scanning microscopy. Science 1990; 248: 73–76.
2. Hendriks RFM, Lucassen GW. Two photon fluorescence microscopy of *in vivo* human skin. Progress in Biomedical Optics and Imaging 2000; 1: 116–121.
3. König K. Multiphoton microscopy in life sciences. J Microsc 2000; 200 (Part 2): 83–104.
4. König K, Peuckert C, Riemann I, Wollina U. Noninvasive 3D optical biopsy of human skin with NIR-femtosecond laser pulses for diagnosis of dermatological disorders. Proc SPIE 2002; 4620: 190–201.
5. Masters BR. Three-dimensional confocal microscopy of human skin *in vivo*: autofluorescence of human skin. Bioimages 1996; 4: 13–19.
6. Masters BR, So PTC, Gratton E. Multiphoton excitation fluorescence microscopy andspectroscopy of in vivo human skin. Biophys J 1997; 72: 2405–2412.
7. Masters BR, So PTC, Gratton E. Multiphoton excitation microscopy of *in vivo* human skin. Ann NY Acad Sci 1998; 838: 58–67.
8. Rajadhyaksha M, Rox Anderson R, Webb RH. Video-rate confocal scanning laser microscope for imaging human tissues *in vivo*. Appl Optics 1999; 38: 2105–2115.
9. Rajadhyaksha M, Gonzalez S, Zavislan JM, Anderson RR, Webb RH. In vivo confocal laser scanning laser microscopy of human skin II: advances in instrumentation and comparison with histology. J Invest Dermatol 1999; 113: 293–303.
10. König K, Riemann I. High-resolution multiphoton tomography of human skin with subcellular spatial resolution and picosecond time resolution. J Biomed Opt 2003; 8: 432–439.
11. König K, Ehlers A, Stracke F, Riemann I. In vivo drug screening in human skin using femtosecond laser multiphoton tomography. Skin Pharmacol Physiol 2006; 19: 78–88.
12. Krutmann J. Skin aging. Hautarzt 2003; 54: 833–838.
13. Schürer NY. Anti-aging. Facts and visions. Hautarzt 2003; 54: 833–838.
14. Koehler MJ, König K, Elsner P, Bückle R, Kaatz M. In vivo assessment of human skin aging by multiphoton laser scanning tomography. Opt Lett 2006; 31: 2879–2881.
15. Lin SJ, Wu Jr R, Tan HY, Lo W, Lin WC, Young TH, Hsu CJ, Chen JS, Jee SH, Dong C. Evaluating cutaneous photoaging by use of multiphoton fluorescence and second harmonic generation microscopy. Opt Lett 2005; 30: 2275–2277.
16. Koehler MJ, Hahn S, Preller, Elsner P, Ziemer M, Bauer A, König K, Bückle R, Fluhr JW, Kaatz M. Morphological skin ageing criteria by multiphoton laser scanning tomography: non-invasive in vivo scoring of the dermal fibre network. Exp Derm 2008; 17: 519–523.

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