

In vivo assessment of human skin aging by multiphoton laser scanning tomography

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Changes of dermal collagen and elastin content are characteristic for skin aging as well as for pathological skin conditions. To evaluate these changes, we used *in vivo* multiphoton laser tomography to measure two-photon excited autofluorescence (AF) and second harmonic generation (SHG). We tested 18 patients of all ages and calculated the SHG-to-AF aging index of dermis (SAAID). We observed a negative relationship between the SAAID and age, which was accelerated for the female ($n=7$) subgroup. The current findings are the first *in vivo* demonstration of this relationship, and they show that specific characteristics of aged skin such as the ratio of extracellular matrix components collagen and elastin can be evaluated by *in vivo* AF and SHG measurements using near-IR femtosecond laser pulses. © 2006 Optical Society of America
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Major features of aged skin include dryness, flaccidness, wrinkling, multiple, mostly benign neoplasms, signs of chronic UV damage, carcinogenesis, and functional deficiency. Skin aging is caused by intrinsic and extrinsic factors. Intrinsic skin aging describes the irreversible physiological process that starts as soon as physical maturation is accomplished. It is characterized by increasing loss of cell number, substance, and function. Extrinsic skin aging is mainly a consequence of cumulative UV exposure of the skin, but can be accelerated by tobacco abuse. Extrinsic and intrinsic skin aging involve similar changes, and extrinsic factors act to aggravate the course of intrinsic skin aging. Moreover, specific alterations such as elastosis cutis and pigment shifts are induced by extrinsic factors. UV exposure is the main cause for premature skin aging.¹

An important clinical parameter for the visible process of skin aging is the dermal collagen content. Collagen I forms 70–90% of dermal extracellular matrix, and its portion decreases by about 1% per year. This decrease is explained mainly by the UV-induced expression of metalloproteases (MMPs) that digest dermal collagen.^{2,3} MMP activity also seems to be induced by nicotine abuse.⁴ The second important hallmark of skin aging is the extensive destruction or

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.³

So far, dermal matrix composition has been evaluated only by histological examination of previously excised skin specimens. To quantitatively evaluate the alterations in skin composition over time it would be necessary to have a tool that allows the *in vivo* assessment of these changes.

Multiphoton laser imaging is a novel tool for the noninvasive evaluation of cellular and molecular structures.⁵ As collagen is able to generate second harmonics and elastin has an excitation maximum in the blue spectral range, both components can ideally be excited at 820 nm by two-photon processes. A filter system can be used for the separate measurement of elastin and collagen because emission of second harmonics is at 410 nm, whereas elastin autofluorescence (AF) has a maximum in the green spectral range.⁶

Recently multiphoton laser imaging has been proposed to quantify the severity of photoaging.⁷ In that work, AF and second harmonic generation (SHG) images of the superficial dermis have been obtained *ex*

Table 1. Characteristics of Subjects

No.	Sex	Age	SAAID ^a	Matched Pairs
1	M	21	0.26±0.03	1
2	F	22	0.21±0.08	1
3	M	26	0.28±0.11	2
4	F	27	0.00±0.06	2
5	F	31	0.19±0.03	3
6	M	37	0.03±0.07	3
7	M	38	0.05±0.12	4
8	F	49	-0.08±0.07	4
9	M	56	0.11±0.05	5
10	F	56	-0.19±0.17	5
11	F	59	-0.18±0.16	6
12	M	66	-0.02±0.08	6
13	M	71	-0.14±0.07	NA
14	M	76	-0.15±0.10	NA
15	M	78	-0.07±0.07	NA
16	F	81	-0.38±0.16	7
17	M	81	-0.22±0.16	7
18	M	84	0.03±0.12	NA

^aSAAID values are presented as means±standard deviation. Equal numbers in the Matched Pairs column correspond to the members of one pair in nonparametric testing for gender differences. Four male test persons left because of unequal gender distribution within the test group. M, male; F, female.

in vivo and found to be correlated with histological findings. A SHG-to-AF aging index of dermis (SAAID) has been proposed. It is defined as $SAAID = (SHG - AF) / (SHG + AF)$, with SHG and AF as the corresponding photon numbers acquired in a rectangular area.

In our study, the assessment of skin aging by means of multiphoton laser imaging with measurement of SHG and AF and determination of the SAAID was performed *in vivo*. Difficulties and pitfalls of this novel technique and applications other than skin aging are discussed in this Letter.

There were 18 adult white European volunteers (7 female) who were recruited from the Department of Dermatology of the Hospital of Friedrich Schiller University in Jena, Germany. The study was performed with informed consent of all participants and was approved by the local ethics committee. We excluded participants whose diseases were suspected to interfere with our measurements. The test group was sampled from all age levels as well as possible. Male and female participants have been age matched to control for the effect of gender on the relationship between age and skin parameters. The sample is described in Table 1.

The CE-marked multiphoton tomograph DermaInspect (JenLab GmbH, Jena, Germany) has been used as described elsewhere.⁸ In brief, the 1M laser device consists of a femtosecond tunable (750–850 nm) Ti:sapphire laser, a galvo- and piezo-scanning system, a photomultiplier tube detection module for time-correlated single-photon counting, and a control module with image-processing hardware and soft-

ware. Scattered laser radiation was blocked with a SP720 filter in front of the detector.

Measurements have been performed at the inner forearm about 15 cm proximal of the wrist. Thus extreme differences in UV exposure of the measuring point have been avoided because of its relatively sun-protected localization, which facilitates the determination of intrinsic skin aging. Furthermore, the inner forearm turned out to be most suitable because of its facile accessibility, little susceptibility to movement artefacts, and low hair density. Five randomly chosen regions just beneath the basement membrane ($z \approx 110 \mu\text{m}$) have been measured twice at an excitation wavelength of 820 nm and with a laser power of 49 mW. SHG images have been obtained by using a 410 ± 5 nm bandpass filter, and AF images have been obtained with a 470 nm long-pass filter. Typically, an area of 128×128 pixels corresponding to $0.2 \text{ mm} \times 0.2 \text{ mm}$ has been imaged with microsecond beam dwell time per pixel.

Photon numbers per pixel for every image have been exported from SPCImage software (Becker & Hickl GmbH, Berlin, Germany) and imported into Microsoft Excel worksheets for SAAID calculation. Test statistics have been performed with SPSS 13.0 (LEAD Technologies).

Multiphoton tomography based on optical sections in the dermis of 18 volunteers has been performed. The SAAID values have been determined based on photon numbers per pixel. The main results are depicted in Fig. 1, in which each person's mean SAAID score, along with its standard deviation, is plotted against the subjects' age. Visual inspection reveals that SAAID scores decline with age. To formally capture this relationship we fitted a linear regression line to describe the data ($R^2 = 0.58$). Nonparametric testing for differences between the eldest six and the youngest six test persons (each representing one third of the test group) showed significant differences ($p < 0.05$) in the SAAID.

Interestingly, the relationship between SAAID and age is modulated by the gender factor. Therefore we fitted separate regression lines for the male and female subgroups (Fig. 2). As can be inferred from the increase in R^2 to 0.89 for the female and 0.68 for the male subgroup, the subjects' sex seems to be an important source of systematic variance. The slope esti-

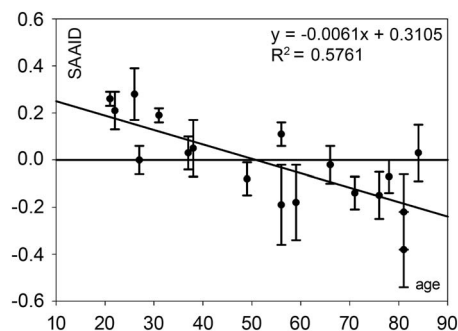


Fig. 1. SAAID distribution depending on the subject's age. Error bars represent the standard deviation resulting from five measurements at five different regions. The regression line demonstrates the SAAID as a function of age.

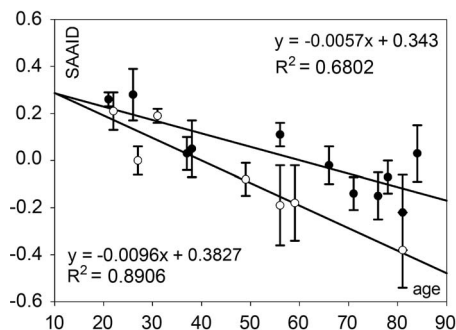


Fig. 2. SAAID distribution depending on the subject's age and sex. Regression lines for either sex separately demonstrate the SAAID as a gender-dependent function of age. Filled circles, male; open circles, female.

mates imply that the decrease in SAAID with age is much more pronounced for women. Additionally, every female test person has been age matched to a male test person (Table 1), and the pairs were tested for differences by the Wilcoxon test, where significance was proven for the hypothesis of a lower SAAID for women ($p < 0.05$).

It was clearly demonstrated that specific characteristics of aged skin, such as the proportion of dermal collagen and elastin content, can be evaluated by *in vivo* multiphoton laser measurements. Thus the decrease of the SAAID with age could have been demonstrated *in vivo*. These findings are consistent with the exemplary *ex vivo* findings of Lin *et al.*⁷ Additionally, we were able to quantify and test the measurements on the basis of our sample and to show gender-specific differences. As these differences are most notably present beyond the reproductive phase, a connection to decreasing sex hormone levels in the context of menopause is plausible. This is consistent with numerous studies that have shown antiaging effects of estrogen and progesterone.⁹

The differences in absolute SAAID values as well as in intra-individual variability in comparison with those presented by Lin *et al.*⁷ are probably due to different optical system characteristics. Furthermore, the SAAID of the data presented here was calculated from two successive measurements, whereas Lin *et al.*⁷ used a beam splitter for simultaneous image acquisition. Thus movement artefacts of the *in vivo* measurements are most probably responsible for the relatively high intra-individual variance in our data.

The interindividual variance, and particularly its apparent increase with age, is probably mainly caused by extrinsic factors, such as sun exposure and smoking habits, which lead to skin damage. However, it might as well be influenced by protective intrinsic

factors, such as sex hormone levels and skin pigmentation.

To compare SAAID values obtained with different multiphoton laser scanning devices, optical instruments and acquisition software should be standardized, or at least appropriate algorithms for mathematical correction should be elaborated.

Further studies are required to define the extent to which the suspected extrinsic factors contribute to skin aging, to assess the physiological curve progression, and to verify the apparent differences between sexes. To validate the new method presented in this pilot study, SAAID measurements should be correlated with data obtained by established biophysical methods that quantify changes associated with skin aging, such as the measurement of elasticity, skin surface hydration, or wrinkle number and depth. Considering the variety of possibly responsible or interfering factors for skin aging, a deliberate selection of test persons is crucial. For example, to evaluate skin aging by solarium visits, the test group has to be restricted or at least matched not only for age and sex, but also for smoking habits and pigmentation. Long-term prospective studies with a large number of healthy volunteers may allow the quantification of the most important influencing factors for the physiological mechanisms that involve changes in dermal collagen or elastin content. Later on, the SAAID may also be used to noninvasively assess the progress of dermatological disorders associated with these changes, such as scleroderma or graft versus host disease. Other future applications of multiphoton laser imaging with determination of the SAAID may be the monitoring of wound healing or of matrix destruction by invasive tumours.

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