New developments in multimodal clinical multiphoton tomography

Karsten König[1,2,3*]
[1]JenLab GmbH, Schillerstr. 1, 07745 Jena, Germany,
[2]Department of Biophotonics and Laser Technology, Saarland University,
Campus A5.1, 66123 Saarbrücken, Germany
[3]Beckman Laser Institute and Medical Clinic, UCI, 1002 Health Science Road, Irvine, CA 92697

ABSTRACT

80 years ago, the PhD student Maria Göppert predicted in her thesis in Göttingen, Germany, two-photon effects. It took 30 years to prove her theory, and another three decades to realize the first two-photon microscope. With the beginning of this millennium, first clinical multiphoton tomographs started operation in research institutions, hospitals, and in the cosmetic industry. The multiphoton tomograph *MPTflex*™ with its miniaturized flexible scan head became the Prism-Award 2010 winner in the category *Life Sciences*. Multiphoton tomographs with its superior submicron spatial resolution can be upgraded to 5D imaging tools by adding spectral time-correlated single photon counting units. Furthermore, multimodal hybrid tomographs provide chemical fingerprinting and fast wide-field imaging. The world's first clinical CARS studies have been performed with a hybrid multimodal multiphoton tomograph in spring 2010. In particular, non-fluorescent lipids and water as well as mitochondrial fluorescent NAD(P)H, fluorescent elastin, keratin, and melanin as well as SHG-active collagen have been imaged in patients with dermatological disorders. Further multimodal approaches include the combination of multiphoton tomographs with low-resolution imaging tools such as ultrasound, optoacoustic, OCT, and dermoscopy systems. Multiphoton tomographs are currently employed in Australia, Japan, the US, and in several European countries for early diagnosis of skin cancer (malignant melanoma), optimization of treatment strategies (wound healing, dermatitis), and cosmetic research including long-term biosafety tests of ZnO sunscreen nanoparticles and the measurement of the stimulated biosynthesis of collagen by anti-ageing products.

Keywords: multiphoton tomography, two-photon, CARS, OCT, ultrasound, optical biopsy, FLIM, Prism award, anti-ageing, collagen, elastin, melanoma, dermatitis, wound healing, skin, tomograph, imaging, skin, nanoparticle, SHG, FLIM, ZnO

1. INTRODUCTION

Maria Göppert (*June 28, 1906 in Kattowitz, - Febr 20, 1972 in San Diego, 1963: Nobel Prize)) predicted in her PhD thesis in Göttingen (Germany) two-photon effects[1,2]. She got married to the young physicist Joseph Edward Mayer who worked in the same department and moved to the United States. In 1960, Maria Goeppert-Mayer became a professor at the new university UCSD in San Diego. It was the year where the laser was invented. Some months later, Wolfgang Kaiser (*July 17, 1925 in Nürnberg) and C.G.B. Garrett demonstrated with this novel intense light source two-photon excited fluorescence in crystals[3]. In 1978, Sheppard and Kompfner suggested to build a non-linear microscope[4]. In 1990, Winfried J. Denk (*Nov 12, 1957 in München), James H. Strickler, and Watt W. Webb realized the first two-photon microscope and performed measurements on living cells[5-6]. One decade later, the multiphoton tomograph DermaInspect™ (JenLab GmbH, Jena, Germany) was developed as first femtosecond laser-based clinical diagnostic system by K. König and co-workers[7-10].

*info@jenlab.de; phone +(49) 3641 470501; fax +(49) 3641 470543; www.jenlab.de

Multiphoton Microscopy in the Biomedical Sciences XI, edited by Ammasi Periasamy, Karsten König, Peter T. C. So,
Proc. of SPIE Vol. 7903, 790305 · © 2011 SPIE · CCC code: 1605-7422/11/$18 · doi: 10.1117/12.874965

In *vivo* multiphoton tomography (MPT) for high-resolution imaging of human skin such as melanoma detection[11], diagnostics of dermatological disorders, cosmetic research[12] and skin aging measurements[13] as well as *in situ* drug monitoring[14], tissue engineering[15], and biosafety tests of ZnO nanoparticles[16] has become a promising method in leading European skin research institutes, clinics in Brisbane, Paris, London, Jena, Berlin, Münster, Modena, Gera, Mannheim and Irvine/California as well as in major cosmetic companies such as L'Oreal and Beiersdorf AG and the major companies in Japan. About 2,000 volunteers and patients have been investigated.
Multiphoton imaging is achieved by focusing femtosecond laser radiation at low picojoule pulse energy into the skin. Intrinsic skin fluorophores, such as elastin, melanin, flavins, keratin, and reduced nicotinamide adenine dinucleotide (NADH), can be used for imaging. Additionally, second harmonic generation (SHG)[17] can be induced to detect the collagen network. Multiphoton tomographs provide optical biopsies with superior submicron resolution. The DermaInspect™ (JenLab GmbH) became the first clinical multiphoton tomograph. In 2010, the second generation and Prism-Award winning flexible tomograph MPTflex™ (JenLab GmbH) has been introduced with its articulated arm and the compact scan head. Safety aspects of these femtosecond laser tomographs for skin imaging in comparison with UV lamp exposure and sun light exposure have been studied by Fischer et al.[18] and other research groups. According to their results, an examination of a skin lesion with the multiphoton tomograph corresponds to light exposure effects similar to a 15 min walk (sun light intensity: 100 mW/cm^2). This review presents a short review on the latest developments including multidimensional MPT and multimodal MPT. First clinical CARS studies have been performed with a novel hybrid multiphoton tomograph.

2. THE PRISM AWARD WINNING TOMOGRAPH MPTflex™

The laser source of the CE-marked clinical multiphoton tomograph MPT*flex*™ (Figs. 1-2) is a sealed turn-key tunable 80 MHz titanium:sapphire femtosecond laser (710 - 920 nm) which is mounted on an optical breadboard. The optical unit consists of an active optical power attenuator to regulate the *in situ* power of the laser in dependence on skin depth, an active beam stabilization device, a safety unit and a flexible articulated mirror-arm with its compact scan head. The scan head consists of a fast galvo-scanning device to generate 2D (XY) scans, a piezodriven z-scanner, and high NA focusing optics (NA 1.3). The optical arm is stabilized with a mechanical arm. The scan head contains also a dual photon detector unit for the measurement of autofluorescence and SHG. The *in situ* power at the skin is 2 mW (stratum corneum) to 50 mW (dermis), the *in situ* laser pulse width 250 fs.
The overall field-of-view of the optical system covers 350 x 350 µm^2. Optical sections can be generated as deep as 200 µm. The image generation is supported by single photon counting (SPC) technology.

Fig. 1. Images of the Prism-Award winning device multiphoton tomograph MPTflex™**.** The scan head consists of a 3D scanning module, the focusing optics, two detectors for fluorescence and SHG imaging, and a 1.7 mm thick microendoscope. Photo (left) taken by Paul Kennedy.

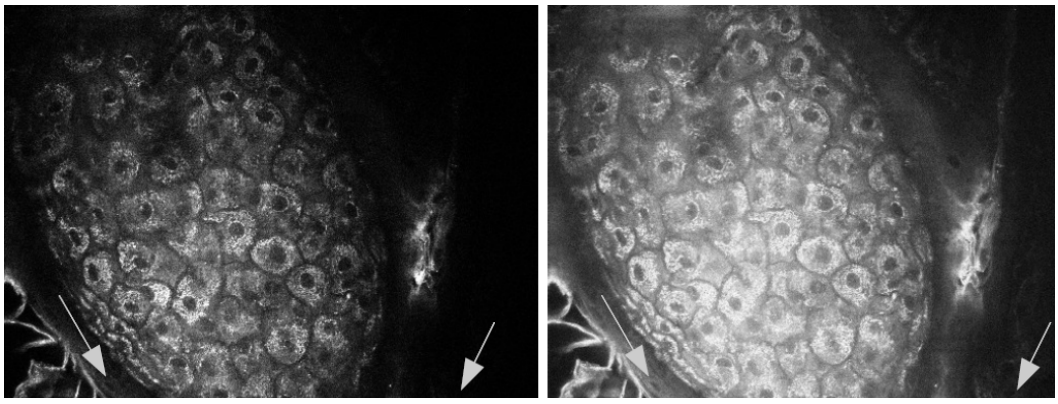


Fig. 2 Typical images in the analog mode and the single photon counting mode.

3. MULTIDIMENSIONAL MULTIPHOTON TOMOGRAPHY

The novel multiphoton tomograph provides label-free 3D optical biopsies based on two-photon imaging of endogenous fluorophores (NADH, melanin, keratin, elastin. flavins) as well as of dermal collagen by SHG. A lateral resolution of <0.5 µm and an axial resolution of 1-2 µm can be achieved inside the tissue. The systems can be upgraded to a fourth dimension by the use of fast photon detectors and time-resolved single photon counting (TCSPC) modules.

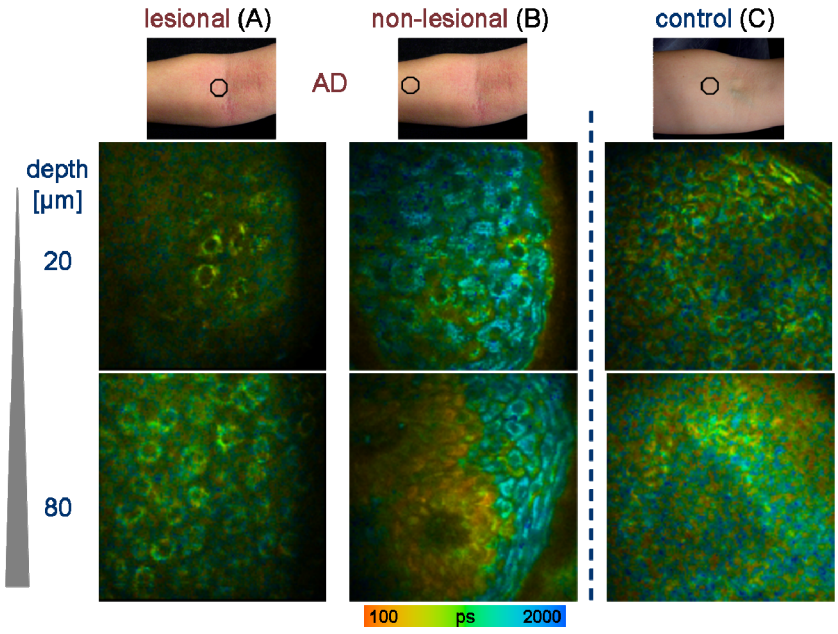


Fig. 3 Clinical Multiphoton FLIM. Researches at the University of Münster, Germany, use the multidimensional multiphoton tomograph for early diagnosis of dermatitis and for the optimization of the treatment (19).

Most multiphoton tomographs are equipped with photomultipliers that provide an instrumental response function of about 250 ps. When using MCPs, the temporal resolution values can be reduced down to 20 ps. TCSPC provides fluorescence decay curves. Fluorescence lifetimes per pixel can be calculated from these curves (512x512 per frame) and biexponential fitting procedures. Finally, fluorescence lifetime images can be generated and depicted as color-coded optical sections. Fluorescence lifetime imaging (FLIM) can be employed for early diagnosis of young patients suffering from dermatitis as well as for treatment control[19], Fig. 3.
A further upgrade is the information on in vivo emission spectra. It is possible to generate spectral information per pixel by employing multiple detector units or by the use of filter wheels in front of the PMT. Researchers in London and Jena use a 4-PMT-approach in combination with beamsplitters to realize spectral FLIM[20,21]. Other research groups use 24PMT-arrays in combination with a polychromator to perform spectral imaging. Fig. 4.

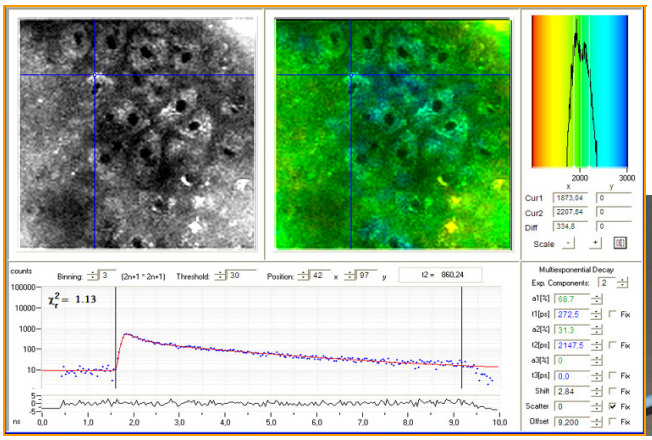


Fig.4a 4D-MPT based on FLIM

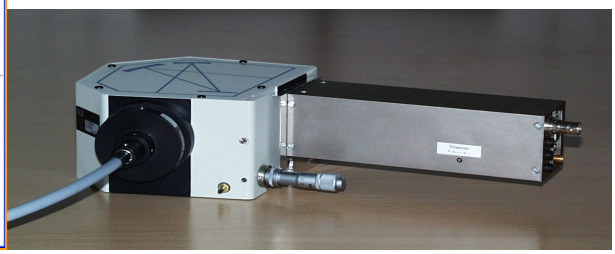


Fig. 4b. 5D-MPT based on spectral FLIM.

3.1. Multimodal MPT using wide-field low-resolution state-of-the-art skin imaging tools

The resolution of a multiphoton tomograph is better than that of the typical non-laser light microscope of a pathologist who investigates 7µm thick HE-stained sections of physically removed skin biopsies. MPT can provide optical biopsies with similar or better information than the conventional HE biopsy. However, multiphoton tomographs provide the subcellular resolution so far just in a small field of view (0.4x04 mm^2) with a maximum signal depth of 0.2 mm. It would be of interest to generate in a first step skin images with a larger field of view and with deep-tissue information. From these images, regions of interests (ROI) should be defined for further high resolution MPT investigations.
Such wide-field tools which have a low resolution compared to multiphoton imaging are color CCD cameras (dermoscopes), ultrasound systems from 7.5-100 MHz, and OCT systems such as the 4-beam swept source OCT for fast 3D skin imaging. We demonstrated the successful combination of MPT with wide-field clinical state-of-the-art systems and performed multimodal imaging a clinical study on patients with different skin diseases in Jena, Germany[22,23]. For example, a swept source 3D OCT system was employed that covered 5 x 5 x 2 mm^3 with a lateral resolution of < 7.5 µm and a vertical resolution of < 10 µm. OCT and MPT images through coverslips with grids and further markers were possible. This allowed the determination of well-defined regions of interests (ROI) within the OCT image and the generation of multiphoton stacks within this ROI.
The Fig. 5 demonstrates the dermoscope system *Fotofinder*, clinical ultrasound systems (7.5 MHz-100 MHz), and an swept-source OCT system. The multimodal approach is seen in the clinical example of a patient suffering from the blister disease *Phemphigus* (Fig. 6).

Fig. 5. The dermoscope Fotofinder, clinical ultrasound systems up to 100 MHz, and a 4beam-swept source OCT present state-of-the-art imaging tools in dermatology.

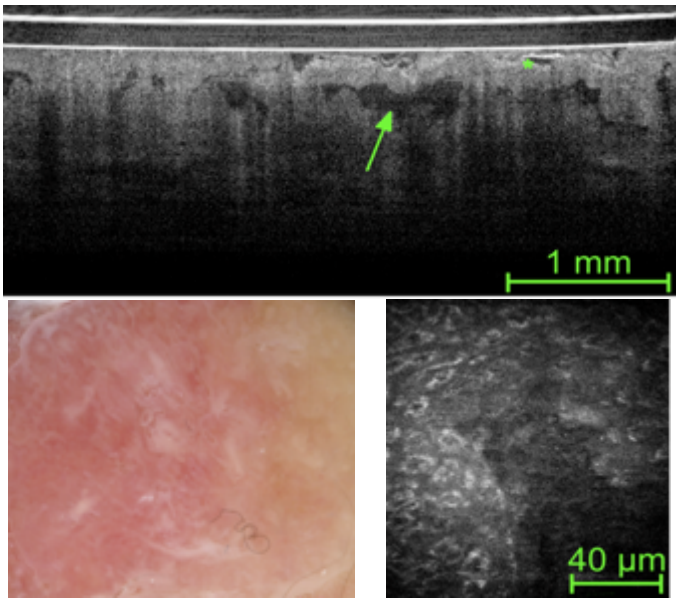


Fig. 6 OCT image, dermoscope image, and multiphoton image of a patient with Phemphigus (11).

3.2. Multimodal MPT – Clinical CARS

Coherent AntiStokes Raman Spectroscopy (CARS) microscopy is a novel imaging technology which has major advantages compared to confocal Raman imaging[24-28]. We performed the first clinical CARS studies by combining the CE-marked clinical product DermaInspect™ with an optical parametric oscillator (OPO, APE Berlin) as well as a time-delay unit. The hybrid CARS/DermaInspect tomograph was employed in a clinical study at the Charite/Berlin in April 2010 after approval by the Certified Body and the local Ethics Committee. Patients suffering from Psoriasis have been studied where modifications of the lipid pattern has been reported[29]. Details of the hybrid systems and the measurements can be obtained from refs. 30-32. Fig. 7 shows an autofluorescence image as well as a CARS image from the non-affected *stratum corneum* of a patient. Fig. 8 demonstrates one example of a lipid-water-emulsion measurement on skin by CARS.

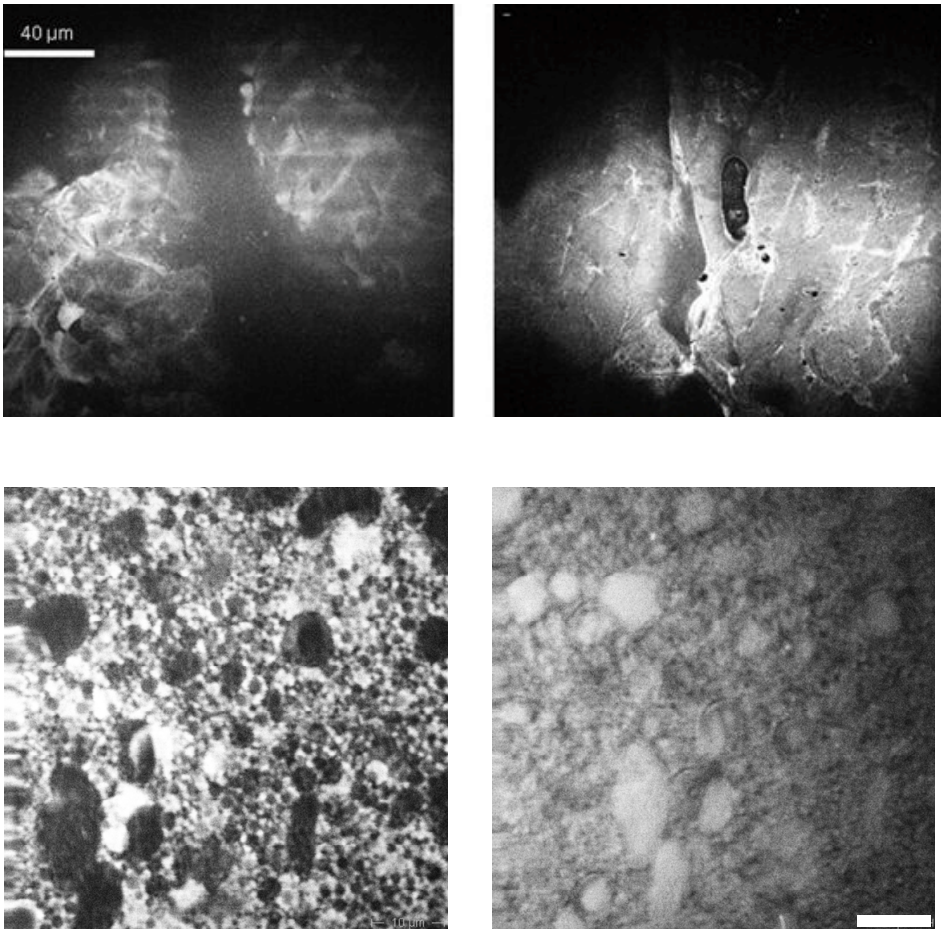


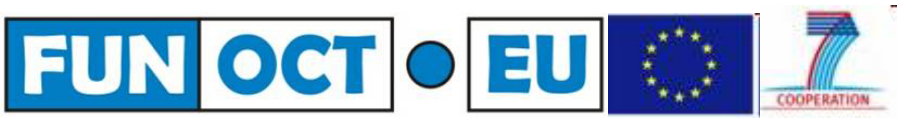
Fig. 7. The novel multimodal hybrid CARS Multiphoton Tomograph provides optical biopsies based on CARS detection of lipids and water, the SHG signal from collagen, and imaging of fluorescent intratissue components. The upper part demonstrates examples of the *stratum corneum* (left: autofluorescence, right: CARS-lipids). The lower part shows CARS images of a water-oil-emulsion.

4. SUMMARY AND CONCLUSION

Clinical CARS has been demonstrated on patients using a novel CE-marked multiphoton hybrid tomograph based on femtosecond NIR laser beams. High-resolution optical biopsies from the human skin were taken with morphological and functional information as well as chemical fingerprinting. In particular, femtosecond CARS provided information on the non-fluorescent and non-SHG active intratissue components lipids and water. Two-photon autofluorescence images provided a detailed view of the surrounding skin architecture and on intracellular morphology whereas SHG detection provided information on the collagen network. Functional imaging can be performed when upgrading the system to a 5D imaging tool with spectral and temporal information of the signals.
In order to get further information on the skin area and skin depth with millimeter dimensions, photoacoustics, acoustics, OCT, and reflection state-of-the-art clinical skin imaging tools can be added to the multiphoton tomograph.
Multimodal multiphoton tomographs have the potential to reduce the amount of physically taken skin biopsies due to their superior resolution, their immediate results within seconds, the absence of fixation and staining procedures, and the chance to perform long-term imaging under physiological conditions. It may become the High-Tech tool for pathologists and dermatologists.

Acknowledgments

I would like to thank the dermatologists Dr. M. Kaatz and Dr. J. Koehler from the Dept. of Dermatology, University of Jena, Prof. S. Schneider, Prof. T. A. Luger and Dr. V. Huck from the University of Münster, Prof. M. Roberts from the Princess Alexandra Hospital in Brisbane, Prof. J. Lademann and Prof. W. Sterry from the Charite at Berlin and the dermatologists, physicists and engineers in London, Modena, Mannheim, Heidelberg, Risoe, Berlin, Magdeburg, Gera, Tokyo, Irvine, San Diego, and Paris for their contributions in the field of clinical multiphoton tomography. Furthermore, I would like to thank the researchers from the company APE in Berlin and Newport/Spectra Physics (Darmstadt, Newport) for supporting the CARS studies.
This work was in part financed by the European Commission (FP 7 program: FUNOCT: combination MPT-OCT and SKINSPECT: combination MPT and ultrasound/photoacoustics), the German Ministry BMBF (combination MPT and CARS) and the American distributer of JenLab products, the MultiPhoton Laser Technologies, Inc.



REFERENCES

[1] Göppert-Mayer, M., „Über die Wahrscheinlichkeit des Zusammenwirkens zweier Lichtquanten in einem Elementarakt“, Die Naturwissenschaften 17, 932 (1929).
[2] Göppert-Mayer, M., „Über Elementarakte mit zwei Quantensprüngen“, Ann. Physik 9, 273-294 (1931)
[3] Sheppard S.J.R, Kompfner R., “Resonant scanning optical microscope”, Appl. Opt. 17, 2879-2882 (1978)
[4] Kaiser, W., Garrett C.G.B., “Two-photon excitation in CaF2:Eu2+,” Phys. Rev. Lett. 7, 229-232 (1961)
[5] Denk, W., Strickler, J.H., and Webb, W.W.W., “Two-photon laser scanning fluorescence microscopy”, Science 248, 73-76 (1990)
[6] Patents US Patent 5034613, July 23, 1991, Japanese Patent 2,848,952 (1998), European Patent 0500717B1 (1998). Licensed to Carl Zeiss.
[7] König K., Patent filed Dec 22, 2000/ DE 10065146 A1
[8] König K., et al., “Optical tomography of human skin with subcellular spatial and picosecond time resolution using intense near infrared femtosecond laser pulses”, SPIE Proceed. 4620, 191-201 (2002)
[9] König K. and Riemann I., "High-resolution multiphoton tomography of human skin with subcellular spatial resolution and picosecond time resolution," J Biomed Opt 8, 432-439 (2003)
[10] König K., "Clinical multiphoton tomography," J Biophotonics 1, 13-23 (2008)
[11] Dimitrow E., Ziemer M., Koehler M.J., Norgauer J., König K., Elsner P. and Kaatz M., "Sensitivity and specificity of multiphoton laser tomography for in vivo and ex vivo diagnosis of malignant melanoma," J Invest Dermatol 129, 1752-1758 (2009).
[12] R. Bazin, F. Flament, A. Colonna, R. Le Harzic, R. Bückle, B. Piot, F. Laizé, M. Kaatz, K. König, J. W. Fluhr, “Clinical study on the effects of a cosmetic product on dermal extracellular matrix components using a high-resolution multiphoton tomography”, Skin Research and Technology 16, 305-310 (2010)
[13] Koehler M.J., König K., Elsner P., Bückle R. and Kaatz M., "In vivo assessment of human skin aging by multiphoton laser scanning tomography," Opt Lett 31, 2879-2881 (2006)
[14] König K., Ehlers A., Stracke F., and Riemann I., "In vivo drug screening in human skin using femtosecond laser multiphoton tomography," Skin Pharmacol Physiol 19, 78-88 (2006)
[15] Schenke-Layland K., Riemann I., Damour O., Stock U.A. and König K., "Two-photon microscopes and in vivo

multiphoton tomographs-powerful diagnostic tools for tissue engineering and drug delivery," Adv Drug Deliv Rev 58, 878-896 (2006)
[16] Roberts M.S., Roberts M.J., Robertson T.A., Sanchez W., Thörling C., Zou Y., Zhao X., Becker W., Zvyagin A.W., "In vitro and in vivo imaging of xenobiotic transport in human skin and in the rat liver", J Biophotonics 1, 478-493 (2008)
[17] Williams R.M., Zipfel W.R. and Webb W.W.W., "Interpreting second-harmonic generation images of collagen I fibrils," Biophys J 88, 1377-1386 (2005)
[18] Fischer F., Volkmer B., Puschmann S., Greinert R., Breitbart W., Kiefer J., Wepf R., „Risk estimation of skin damage due to ultrashort pulsed focused near infrared laser radiation at 800 nm," JBO 13, 041320-1 (2008)
[19] Huck V., et al., „Intravital multiphoton tomography as an approriate tool for non-invasive in vivo analysis of human skin affected with atopic dermatitis", Proceedings of the SPIE 7883A-26 (2011), in press.
[20] Patalay R., et al., „Fluorescence lifetime imaging of skin cancer", Proceedings of the SPIE 7883A-9 (2011), in press.
[21] Talbott C.B., et al., „A multispectral FLIM microscope for in vivo imaging of skin cancer", Proceedings of the SPIE 7903-80 (2011), in press.
[22] König K., Speicher M., Bückle R., Reckfort J., McKenzie G., Welzel J., Koehler M.j., Elsner P. and Kaatz M., "Clinical optical coherence tomography combined with multiphoton tomography of patients with skin diseases", J Biophotonics 2, 389-397 (2009)
[23] König K., Speicher M., Köhler M.J., Scharenberg R., and Kaatz M., "Clinical application of multiphoton tomography in combination with high-frequency ultrasound for evaluation of skin diseases" J. Biophotonics 3, 759-773 (2010)
[24] Zumbusch A., Holtom G.R. and Xie X.S., "Three-Dimensional Vibrational Imaging by Coherent Anti-Stokes Raman Scattering," Phys. Rev. Lett. 82, 4142 (1999)
[25] Evans C.L., et al., "Chemical imaging of tissue in vivo with video-rate coherent anti-Stokes Raman scattering microscopy," Proc Natl Acad Sci U S A 102, 16807-16812 (2005)
[26] Volkmer A., Cheng J.X., and Xie X.S., "Vibrational Imaging with High Sensitivity via Epidetected Coherent Anti-Stokes Raman Scattering Microscopy," Phys. Rev. Lett. 87, 023901 (2001)
[27] Freudiger C.W., et al., "Label-Free Biomedical Imaging with High Sensitivity by Stimulated Raman Scattering Microscopy, " Science 322, 1857-1861 (2008)
[28] De Beule, P.A.A., Dunsby C., Galletly N.P., Stamp G.W., Chu A.C., Anand U., Anand P., Benham C.D., Naylor A. and French P.M.W., „A hyperspectral fluorescence lifetime probe for skin cancer diagnosis," Rev Sci Instrum 78, 123101 (2007)
[29] Osada M., Gniadecka M. and Wulf H.S., "Near-infrared Fourier transform Raman spectroscopic analysis of proteins, water and lipids in intact normal stratum corneum and psoriasis scales," Exp. Dermatol. 13, 391-395 (2004)
[30] König K., Breunig H.G., Bückle R., Kellner-Höfer M., Weinigel M., Büttner E., Sterry W. and Lademann J., "Optical Skin Biopsies by clinical CARS and Multiphoton Fluorescence/SHG Tomography", Laser Physics Letters. In press (2011)
[31] Breunig H.G., et al., "Combining multiphoton and CARS microscopy for skin imaging", Proceedings of the SPIE 7903, in press (2011)
[32] Breunig H.G., et al., "Combined *in-vivo* multiphoton and CARS imaging of healthy and disease-affected human skin", Submitted.