Quantitative Multiphoton Imaging

Karsten König[1,2], Martin Weinigel[1], Hans Georg Breunig[1,2], Aisada Uchugonova[1,2]
[1]JenLab GmbH, Schillerstr. 1, 07745 Jena, Germany,
[2]Department of Biophotonics and Laser Technology, Saarland University,
Campus A5.1, 66123 Saarbrücken, Germany

ABSTRACT

Certified clinical multiphoton tomographs for label-free multidimensional high-resolution *in vivo* imaging have been introduced to the market several years ago. Novel tomographs include a flexible 360° scan head attached to a mechano-optical arm for autofluorescence and SHG imaging as well as a CARS module. Non-fluorescent lipids and water, mitochondrial fluorescent NAD(P)H, fluorescent elastin, keratin, and melanin as well as SHG-active collagen can be imaged *in vivo* with submicron resolution in human skin. Sensitive and rapid detectors allow single photon counting and the construction of 3D maps where the number of detected photons per voxel is depicted. Intratissue concentration profiles from endogenous as well exogenous substances can be generated when the number of detected photons can be correlated with the number of molecules with respect to binding and scattering behavior. Furthermore, the skin ageing index SAAID based on the ratio elastin/collagen as well as the epidermis depth based on the onset of SHG generation can be determined.

Keywords: multiphoton tomography, two photon, CARS, skin, imaging, optical biopsy, collagen, elastin, NADH, melanin, femtosecond laser, single photon counting, quantitative imaging, concentration maps

1. INTRODUCTION

In vivo clinical multiphoton tomography[1-3] based on two-photon autofluorescence (AF) and second harmonic generation (SHG) for high-resolution label-free imaging of human skin especially for cancer detection[4], diagnostics of dermatitis, cosmetic research[5] and skin aging measurements[6-9] as well as *in situ* drug monitoring[10,11] and tissue engineering[12] has become a promising novel technology. Coherent anti-Stokes Raman Spectroscopy (CARS) has been recently introduced as an add-on module to the certified clinical tomographs[13-15].

Multiphoton imaging is achieved by focusing femtosecond laser radiation at low picojoule pulse energy into the skin. Typically, tunable 80 MHz Ti:sapphire lasers are used. Intrinsic fluorophores, such as elastin, melanin, flavins and reduced nicotinamide adenine dinucleotide (NADH), are naturally part of the skin and can be used for imaging. The excitation of these biomarkers with different laser wavelengths reveals the morphological structure of the skin and chemical fingerprinting. Additionally, SHG can be induced to detect the collagen network. Advantages of clinical multiphoton imaging are the ability to provide superior optical sectioning at depths down to 200 µm without any destructive effect or labeling and the possibility to study long-term effects / pharmacokinetics in a natural physiological environment.

So far, multiphoton autofluorescence/SHG/CARS sections provide intensity signals per pixel resulting in nice high-resolution images. However, it is highly demanded to extract parameters for skin characterization and to provide intratissue concentrations of endogenous molecules such as the reduced coenzyme NAD(P)H and melanin as well as of intratissue cosmetics and pharmaceutics.

When using analog detection systems, the offset and gain of the photomultipliers determine the 8bit intensity signal value per pixel/voxel. In contrast, single photon counting (SPC) provides signals as counts per pixel/voxel. It is desired to detect 1 count or less per laser pulse. With typical beam dwell times per pixel (pp) of 10 µs, maximal 800 counts pp can be generated.

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

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A special type of SPC is time-correlated SPC (TCSPC) where the arrival time of the detected photon with respect to the fs excitation pulse is recorded. FLIM/TCSPC was introduced in Life Sciences by König et al. in 1988/1989[16-17].

2. EXPERIMENTAL DESCRIPTION OF THE MPTFLEX TOMOGRAPHS

The certified clinical multiphoton tomographs MPT*flex*™ and MPT*flex*™-CARS (Fig. 1) consist of a turn-key 80 MHz tunable titanium: sapphire 100 femtosecond laser as excitation source operating in the spectral range of 710 nm – 920 nm. The laser is part of an optical unit that is mounted on a stable optical carbon breadboard to reduce vibrations. The optical unit consists of an optical power attenuator to regulate the output-power of the laser, a special beam stabilization device and a safety unit to prevent tissue damage. A flexible articulated mirror-arm, which is optimized for infrared radiation, guides the laser beam to the scan head. The optical arm I is connected to a mechanical arm with "freezing position" option. 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). To assure laser safety the maximum output power of the laser radiation is limited to 50 mW. The scan head contains also either (i) a miniaturized dual photon detector unit or a miniaturized four detector unit (ii) when operating in the additional CARS mode (Fig. 2). One detector with fast rise time measures the autofluorescence (AF) with 200 ps temporal resolution, the second detector the collagen network by SHG detection. The third detector measures CARS, the fourth detector is customer-specific designed.
The overall field-of-view of the optical system covers 350 x 350 μm^2 but can be increased up to 4x4 mm^2 by tiling (mosaic). Optical sections can be generated as deep as 200 µm according to the demand of the European Certified Bodies. The dual/four channel detection device is supported by a controller box, which additionally controls the z-movement of the focusing optics, the 2D scanning process and the image generation. The intensity per pixel is stored as 16-bit-grey value and processed by the JenLab control software. The image generation is supported by a novel single photon counting (SPC) hardware to improve image quality. The SPC mode, in contrast to the analog (current) mode, counts the number of detected photons and depicts them as gray-scale value of each pixel (Fig. 3).

Fig. 1 Image of the Prism-Award winning clinical multiphoton tomograph MPT*flex*™ for autofluorescence, FLIM and SHG imaging (left) and the novel tomograph MPT*flex*™- CARS for additional CARS imaging (right).

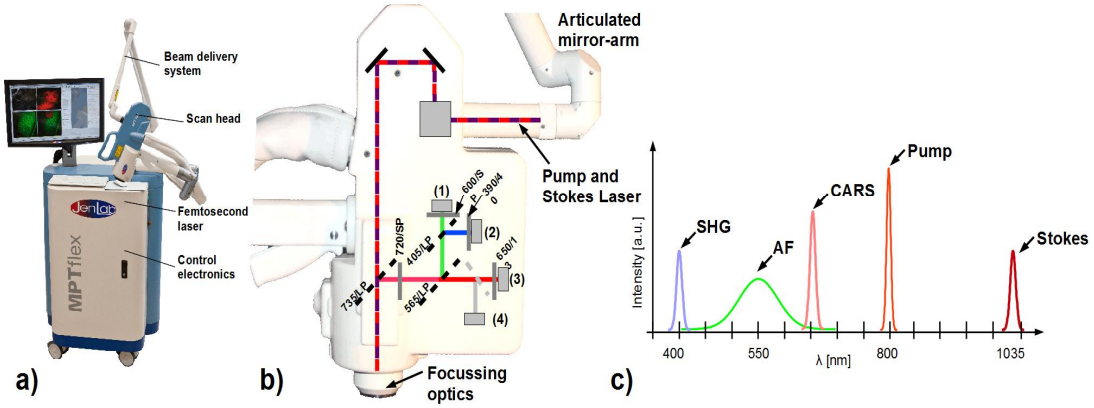

Fig. 2 Multiphoton tomograph (a) with scan head with four-detector arrangement (b) and spectrum of excitation and detection signals (c)[18].

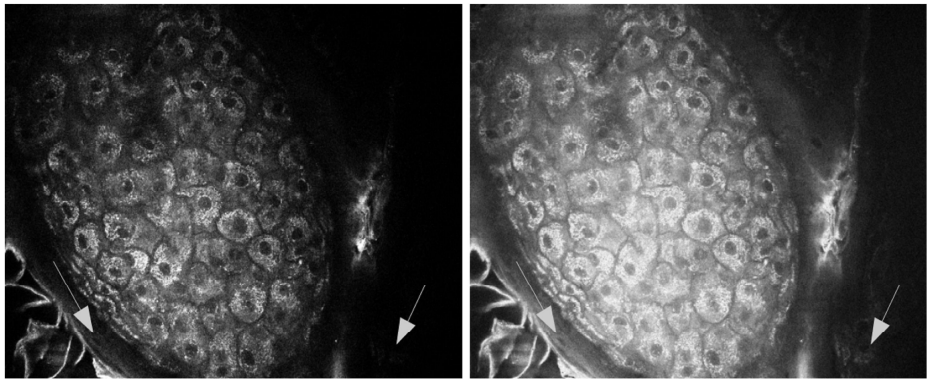

Fig. 3 Typical images in the analog mode and the single photon counting mode (digital mode).

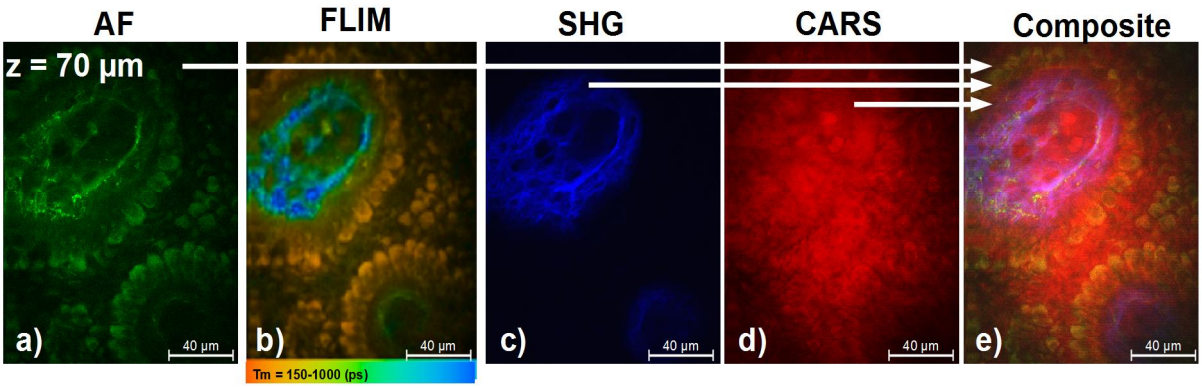

Fig. 4 The multiphoton/CARS tomograph MPT*flex*-CARS with its 4-detector unit provides within one optical section simultaneously autofluorescence (AF), FLIM, SHG, CARS, and overlay information[18].

3. RESULTS AND DISCUSSION

3.1. Analog and digital imaging

The photon detectors provide analog images as well as digital images based on SPC. The quality and the grey levels depend on the offset (threshold) and the gain (voltage) of the photon multiplier. Typically 8bit or 16bit values are provided. The digital mode employs the counts provided by the JenLab SPC card or the TCSPC module. In TCSPC, the counts (arrival times of the signal photons) are listed in a 128 or 256 time channel histogram pp.

Typically, mean power of 2 mW is applied to the upper skin layer, the *stratum corneum*. When going deeper, the incident laser power goes up to 20 mW.

2 mW laser mean power at a wavelength of 780 nm and photon energy of 1.59 eV, respectively, corresponds to $8x10^{15}$ laser photons per second. That means, about 1,840 laser pulses and $2x10^{11}$ laser photons, respectively, are applied per pixel (pp) when using a typical beam dwell time of 23 µs (80 MHz, 7.4 s per frame, 512x512 pixels). The theoretical beam size d= 0.61λ/NA=366 nm corresponds to ½ pixel when using a NA1.3 focusing optics that covers with a typical optical zoom a FOV of 0.3x0.3 μm^2. Practically, the laser focus covers about one pixel.

The NA1.3 optics collect maximal 50% of the AF photons and other signals (SHG, CARS) (Fig. 4) in the backward direction ("epifluorescence"). Assuming additional losses due to transmission/reflection within the detection system of 0.5 and a quantum efficiency of the photon detector of 0.06, the collection efficiency can be estimated to be 1%. With a low fluorescence quantum yield of endogenous fluorophores of about 1%, 20 millions AF photons can be theoretically expected to be counted per pixel during the beam dwell time.

However, TCSPC with its Poisson statistics based deconvolution works well at maximum count rates of 1 count per laser pulse, meaning maximum 1,840 counts per pixel. Because the pulse-pulse temporal distance is 12 ns but the dead time of the TCSPC module about 0.1 µs, maximum 400 counts pp can be expected.

When using TCSPC, typically 128 time channels are used and a bi-exponential fitting procedure of the fluorescence decay curve is employed.

In order to increase the number of counts, the number of pixels can be reduced, e.g. 128x128 pixels, or binning can be employed where the counts of the neighbor pixels are summarized (binning 1: pixel + 8 neighbor pixels). The laser power cannot be increased significantly due to medical product restrictions.

Fig. 5 demonstrates an example of an optical in vivo skin section acquired in TCSPC mode. A binning of 1 and 128x128 pixels were chosen. The bi-exponential fitting of one + neighbor pixels (blue cross) with χ^2=1.02 is excellent. The two fluorescent compounds are free NAD(P)H (short lifetime: 450 ps, 63%) and bound NAD(P)H (long lifetime: 2.5 ns, 37%). The image shows also the total number of photon counts of the 9 pixels, namely 10,375. The mean value per pixel would be 10,375:9=1,153.

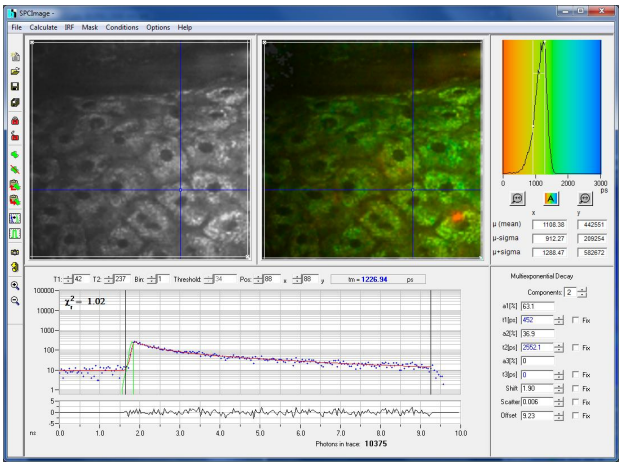

Fig. 5 Optical section of in vivo human skin with fluorescence lifetime information and counts of 9 pixels = binning 1 (n=10,375).

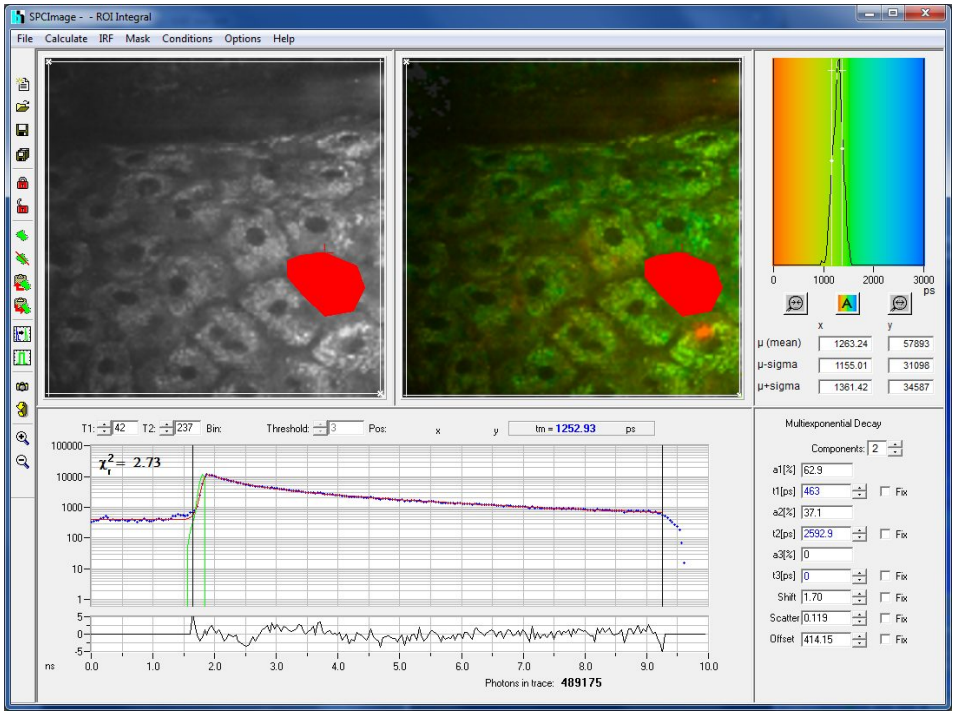

Fig. 6 The software allows the evaluation of regions of interest, e.g. a single skin cell. The number of counts and the number of pixels per ROI can be provided.

In principle, the following parameters have been determined from solutions, cell monolayers, phantoms, tissue biopsies, and in vivo skin. Fig. 6 shows an example of a region of interest (ROI).

counts per pixel: cpp
counts per frame: cpf
counts per ROI (cell): cROI
pixel per ROI: pROI e.g. pROI (128x128): 16,384

The table shows results from solutions/powders. The buffer TRIS served as a control. Values of less than 1 cpp (0.99@760 nm, 0.15@800nm, 0.18@900nm) have been obtained with the exposure parameters 128x128 pixels, 8 mW, 13.4 s, z=20 μm). The 760 nm measurements in NADH solutions resulted in cpf numbers (dark subtracted) of 92,750 (1 mM), 9,017 (0.1 mM), and 2,364 (0.01 mM) with a SNR of 1.15. When adding the protein alcohol dehydrogenase (ADH), the fluorescence quantum yield increased significantly.

Table: CPP values of solutions, powders, and single skin cells.

Substance	**Mean CPP**
TRIS (control)	0
NADH (100 μM)	1
NADH-ADH (100 μM)	195
FAD (100 μM)	2
Melanin (0.1%)	8
Riboflavin (100 μM)	18
Keratin	30
Fibroblast	140 (max: 477)

4. SUMMARY AND CONCLUSION

Clinical multiphoton hybrid 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.

The most exciting hybrid multiphoton tomograph is a two-beam multimodal system that provides 3D optical biopsies with morphological and functional information as well as chemical fingerprinting. In particular, CARS provide information on non-fluorescent and non-SHG active components such as lipids, water and a variety of cytostatic agents. Two-photon autofluorescence images provide perfect information on the skin architecture and the intracellular morphology whereas SHG detection enables the high-resolution imaging of the collagen network. CARS adds to this morphological information.

Furthermore, the coenzymes NAD(P)H and flavins/flavoproteins act as metabolic indicators. The ratio of free to bound NAD(P)H as well as the ratio NAD(P)H/flavins can be determined by TCSPC. This enables functional imaging[19].

SPC hardware and TCSPC modules open the chance to perform quantitative multiphoton imaging by providing photon counts per pixel or per region of interest. Concentration maps can be generated with correlation procedures. These correlations require measurements of solutions/powders/cell monolayers. Furthermore, skin phantoms, biopsies and sufficient in vivo data are required to account for scattering phenomena including reabsorption beam distortion etc. This data analysis is currently performed in our research group in cooperation with bioinformatics partners.

A major point of interest is the definition of a skin ageing parameter based on SPC measurements. The skin age correlates with the ratio of elastin and collagen, the structure of the epidermal-dermal junction as well as the NAD(P)H level in the skin layer *stratum granulosum*. Based on these data, the skin ageing parameters SAAID, ELCOR and others have been defined[6-9]. Quantitative imaging provides a further step in the characterization of skin phenomena and the early multiphoton diagnosis of skin cancer[20].

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