A novel clinical multimodal multiphoton tomograph for AF, SHG, CARS imaging, and FLIM

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ABSTRACT

We report on a flexible nonlinear medical tomograph with multiple miniaturized detectors for simultaneous acquisition of two-photon autofluorescence (AF), second harmonic generation (SHG) and coherent anti-Stokes Raman scattering (CARS) images. The simultaneous visualization of the distribution of endogenous fluorophores NAD(P)H, melanin and elastin, SHG-active collagen and as well as non-fluorescent lipids within human skin in vivo is possible. Furthermore, fluorescence lifetime images (FLIM) can be generated using time-correlated single photon counting.

Keywords: CARS, multiphoton tomography, skin imaging, lipids, SHG, FLIM, two-photon

1. INTRODUCTION

In vivo multiphoton tomography[1, 2] is realized by focusing near infrared (NIR) femtosecond laser pulses inside the tissue and collecting signals in epi-direction. The nonlinear dependence on the excitation intensity minimizes out-of-focus excitation and provides optical sectioning capability with high signal to noise ratios[1-5]. High photon flux densities are achieved by tight focusing (high NA) of low-power laser beams. The use of NIR laser radiation results in reduced out-of-focus bleaching and high penetration depth due to low scattering and low absorption of the skin tissue[1]. The spatially resolved two-photon fluorescence (autofluorescence - AF) of intrinsic fluorophores, such as elastin, melanin, flavines and NAD(P)H reveals the morphological structure of the skin[2]. A further separation of these fluorophores can be realized by time-correlated single photon counting (TCSPC)[6] to perform fluorescence lifetime imaging (FLIM)[3, 7].

In addition to AF, second harmonic generation (SHG) can be induced by excitation-light interaction with non-centrosymmetric protein structures of the dermal collagen network[8].

Optical sectioning capability is also provided by coherent anti-Stokes Raman scattering (CARS) due to its nonlinear nature[9]. CARS allows to access non-fluorescent intra-tissue substances such as lipids[9-12] and water[13]. CARS contrast can be generated by providing a pump (ω_{pump}), a Stokes (ω_{Stokes}) and a probe (ω_{probe}) photon, respectively[14]. To simplify CARS implementation often pump and probe photons are generated by a common light source ($\omega_{pump} = \omega_{probe}$). In case the frequency difference between ω_{pump} and ω_{Stokes} matches the molecular frequency of the vibration of the molecule the CARS signal at $2\omega_{pump} - \omega_{Stokes} = \omega_{CARS}$ is strongly enhanced[14].

An ubiquitous group of naturally existing molecules in human skin are lipids. They are available for instance inside the epidermis as a part of the skin barrier (cholesterol)[15] or inside the cellular membrane (phospholipids) but they also occur inside the dermis - stored inside adipocytes as energy reservoir[16]. Lipids are non-fluorescent, however, rich in Raman-active CH_2 groups with a stretch vibrational transition at 2845 cm^{-1} [10].

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Recently, two clinical certified AF/SHG-CARS tomographs based on optical parametric oscillator (OPO) as well as photonic crystal fiber (PCF) technology for frequency conversion have been developed by us. First clinical results are published[17-19]. Due to the limited number of detectors of these tomographs a separation of simultaneous generated AF and SHG-signals during CARS imaging was not possible. Thus skin aging studies based on SAAID measurements[20] were limited to non-CARS tomographs. In this article, the first demonstration of a compact multichannel-(FLIM)-detector for the clinical tomograph *MPTflex CARS* with its flexible optomechanical arm and compact scan head is described and first *in vivo* measurements are presented.

2. MATERIAL AND METHODS

The certified clinical tomograph *MPTflex* as a mobile flexible nonlinear imaging system simplifies *in vivo* AF/SHG imaging of human skin[21-24]. An articulated optical mirror-arm with active beam position control for free-space-beam delivery between laser and scan head allows a high degree of freedom for positioning of the scan head which is inevitable for a variety of medical applications (Figure 1a). For nonlinear excitation a tunable laser source (Ti:sapphire femtosecond oscillator, MaiTai XF-1, Spectra Physics, USA) is used. The scan head of the tomograph *MPTflex* typically contains a dual-channel detector for simultaneous detection of AF and SHG-signals. Its laser-scanning arrangement allows to acquire AF/SHG-images up to a depth of 200 µm with a field-of-view (FOV) of 350 x 350 µm^2 and a resolution of 0.3-0.6 µm laterally and 1-2 µm axially[2].

For additional CARS modality, the basis system MPT*flex* has been equipped with a compact and stable optomechanical subassembly which includes a photonic crystal fiber (PCF) for the generation of the Stokes pulses and a delay line. The collinearly aligned Stokes- and pump pulses are guided in free space through the articulated optical mirror-arm into the scan head and are focused by a high-NA optics into the tissue.

To realize CARS contrast at ~ 2845 cm^{-1} (which e.g. corresponds to CH_2-symmetric stretch vibrations of lipids) the pump pulses of the femtosecond oscillator are tuned to 800 nm. The Stokes pulses are spectrally narrowed by a band-pass filter (HQ1045-30, Chroma, USA) within the CARS module to select a spectral range of 30 nm (FWHM) out of the PCF broadband continuum. The CARS signal (~651 nm) is detected in epi-direction by the same focusing optics, similar to the detection of AF/SHG signals (Figure 1c).

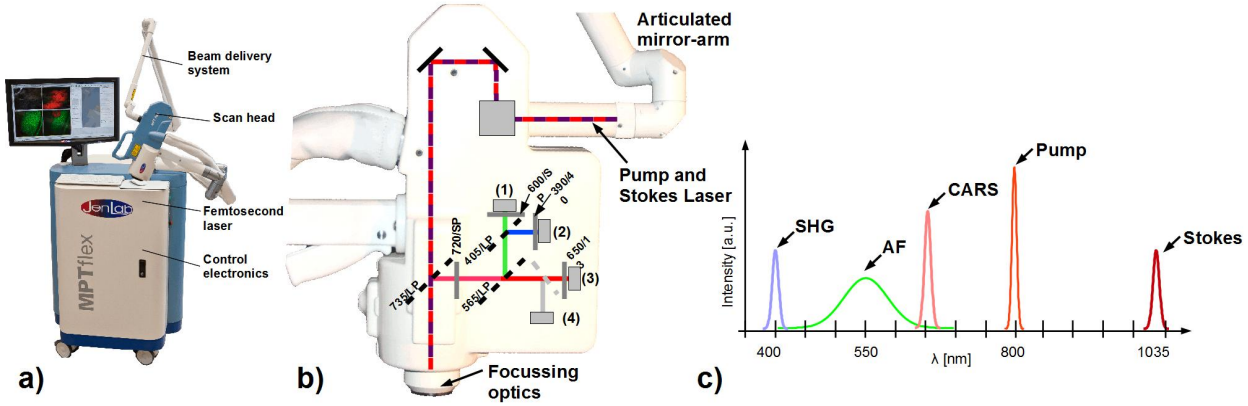

Figure 1: a) Flexible multimodal tomograph MPTflex CARS; b) Scan head with four-detector arrangement; c) Schematic spectrum of excitation and detection signals.

For simultaneous detection and separation of AF/FLIM, SHG and CARS signals a compact four-channel detection module has been developed, which fits into the scan head of the tomograph *MPTflex CARS* (Figure 1b). Compared to previous detector designs, several advantages are realized:

1. Equal beam path lengths for all detection channels.
2. Fast temporal responses of the detectors and the corresponding electronics. The transient time spread (tts) is determined to be better than 150 ps, which is essential for FLIM.
3. Lightweight and tight mechanical design that is insensitive against ambient light.
4. Changeable filter cubes for a customized setup.

Short pass filters are used to separate signal light from residual back-scattered excitation light. A longpass dichroic mirror 565 separates an AF/SHG- and a CARS-channel (3), respectively. The CARS detection is further narrowed to 13 nm (FWHM) centered at 650 nm. The AF/SHG beam path is split by a longpass 405 into a AF-channel (1) equipped with a shortpass 600 to block the residual CARS signals. A 390/40 bandpass in front of the SHG-channel (2) blocks residual AF signals. The optical crosstalk of the AF- and SHG-channel was tested with the strong SHG-signal induced in urea crystals and was found to be negligible. Channel (4) is not employed in this setup but offers the possibility to detect further signals.

The simultaneous readout of all detectors by default is accomplished by single photon counting (SPC) hardware (JenLab, Germany) generating images of 512 x 512 pixels at a mean dwell time of 26 µs/pixel. Additionally, for lifetime measurements the readout of channel (1) is simultaneously recorded by TCSPC hardware.

In CARS geometry the FOV is about 250 x 250 µm². To assure laser safety the total output power of both beams ($P\omega_{pump}$+ $P\omega_{Stokes}$) is limited to 50 mW. For CARS imaging, a coverslip (thickness: 170 µm) and immersion oil is placed in between skin and focusing optics. Water is used as contact agent between skin and coverslip.

3. RESULTS AND DISCUSSION

Validation of FLIM system

FLIM-capability of the lifetime measuring system has been verified by acquiring the lifetime of fluorescein solution (Fluorescein SE Thilo 1,7 mg/ml Fluorescein-Natrium; Alcon Pharma GmbH, Germany). To suppress self-quenching the initial concentration of the fluorescein (0,17%) has been reduced to a concentration of ~0,002%.

The fluorescence decay curve of fluorescein (Figure 2c) reveals a mono-exponential decay function (settings: mono-exponential best-fit curve; 3-fold binning). The decay time distribution of the image in figure 2a) (128 x 128 pixels) is shown in figure 2b). It has a maximum at approximately 4.4 ns. In the literature decay times ranging from 4.1 ns[25] to 4.5 ns[26] are reported in good agreement with our result.

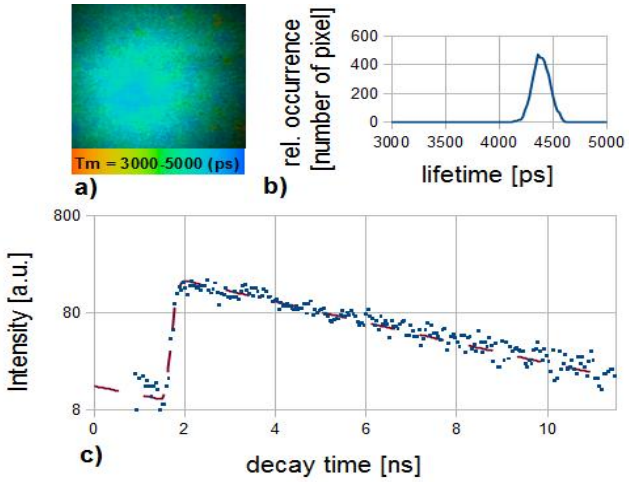

Figure 2: a) 2D lifetime distribution of fluorescein solution, image size: 100 x 100 µm²; b) lifetime distribution histogram; c) representative decay function acquired in the center of a) (measurement data (dotted values), mono-exponential fit (dashed)).

In vivo multichannel imaging

For the demonstration of multichannel AF/FLIM-SHG-CARS functionality, healthy human skin of the forearm of volunteers was imaged *in vivo* at a depth of 70 µm (dermal papillae). The AF channel in figure 3a) represents a circular arrangement of basal cells with elastin fibers in the center. The FLIM matrix in figure 3b) reveals a heterogeneity of lifetimes which is the result of different endogenous fluorophores and their microenvironments within the skin (settings: double-exponential best-fit, 3-fold binning). The basal cells are nicely distinguished from the elastin due to their high content of melanin[2]. Melanin has a strong short lifetime component (orange color coded) and elastin longer lifetimes (blue-green color coded)[7]. The SHG channel in c) visualizes the corresponding interweaved collagen fibers. No AF crosstalk is visible in c). The CARS channel in d) shows sharply separated cellular details but also roundish structures of dermal papillae are noticeable. The composite image of figure 3a), c) and d) is shown in figure 3e).

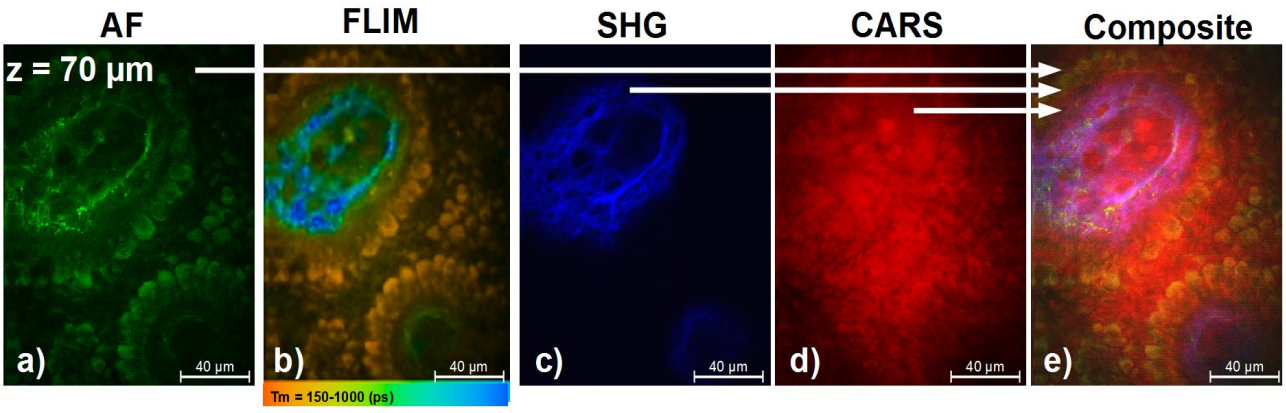

Figure 3: Multichannel imaging of human skin (forearm) *in vivo* at dermal papillae; bar: 40 µm.

The ratio of resonant CARS to non resonant background is determined in vitro by imaging polystyrene microspheres in agarose gel. A ratio of > 5:1 (data not shown here) allows sufficient CARS CH_2 contrast for skin analysis. The lateral and axial resolutions of the CARS modality were determined to be approximately ~0.29 µm and 1.7 µm, respectively.

4. CONCLUSION

Multiphoton tomography allows high-resolution tissue imaging based on autofluorescence and SHG. Supplemental information on non-fluorescent and non-SHG active tissue components can be obtained by CARS. CARS imaging is achieved by the combination of a femtosecond oscillator and a photonic crystal fiber. With the novel and compact multichannel detector, simultaneous detection and separation of AF/FLIM, SHG and CARS signals is possible. The acquired images present the functionality of this novel detector for SPC imaging as well as TCSPC imaging on human skin in vivo with image qualities comparable to previous detectors of the tomograph *MPTflex*.

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