High (1 GHz) repetition rate compact femtosecond laser: A powerful multiphoton tool for nanomedicine and nanobiotechnology

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Multiphoton tomography of human skin and nanosurgery of human chromosomes have been performed with a 1 GHz repetition rate laser by the use of the commercially available femtosecond multiphoton laser tomograph DermaInspect as well as a compact galvoscanning microscope. We performed the autofluorescence tomography up to 100 μm in the depth of human skin. Submicron cutting lines and hole drillings have been conducted on labeled human chromosomes. © 2007 American Institute of Physics. [DOI: 10.1063/1.2745367]

I. INTRODUCTION

As the development of laser sources progresses, their possible applications for biomedical applications have to be evaluated. Due to the optical window in the near-infrared (NIR) region, femtosecond Ti:sapphire lasers are the ideal sources for in vitro and in vivo multiphoton microscopies of biological tissue. Multiphoton autofluorescence imaging of endogenous fluorophores inside the human tissue with NIR femtosecond lasers for the diagnosis of skin is an established method.¹⁻⁶ It permits a painless examination of patients without the necessity to introduce narcotics and fluorescent dyes. Photons absorbed via multiphoton excitation can induce autofluorescence based on naturally occurring endogenous fluorescent biomolecules such as flavines, reduced nicotinamide adenine dinucleotide(phosphate) [NAD(P)H], coenzymes, metal-free porphyrins, components of lipofuscin, melanin, elastin, and keratin.⁴ Second harmonic generation (SHG) of collagen is a further process that provides an accessible information.⁷

Conventional laser sources used for multiphoton microscopy have typical repetition rates in the megahertz range with pulse lengths of 70–170 fs. It was shown by Chu et al.⁸ that Ti:sapphire lasers with repetition rates in the gigahertz regime are promising tools for imaging applications. In contrast to conventionally used femtosecond lasers in the NIR, gigahertz lasers offer the advantage of higher number of pulses while keeping the peak intensity at the same level. This provides a higher signal intensity without increasing photodamage. Furthermore, it allows reducing the peak intensity and by that possible damaging effects while conserving the ability to produce high-resolution multiphoton imaging without staining.

As well, near-infrared femtosecond laser pulses are tools for ultraprecise intracellular and intratissue surgery, cell isolation, and nanostructuring of biomaterials.⁹⁻¹⁴ The required high transient laser intensity of TW/cm² for multiphoton ablation can be achieved with low nanojoule energy laser pulses when using focusing optics of high numerical aperture.

In this work we performed experiments with a 1 GHz repetition rate laser for multiphoton tomography of human skin by the use of a commercially available femtosecond multiphoton laser tomograph and for nanosurgery of chromosomes.

II. MATERIALS AND METHODS

A. Instrumentation

We used a compact (18×40×10 cm³) Ti:sapphire femtosecond laser oscillator with a high repetition rate of 1 GHz with a continuous tuning range from 750 to 850 nm of the central wavelength¹⁵,¹⁶ (GigaJet 20c, prototype version Gigaoptics GmbH, Germany). The oscillator delivers pulses of about 60 fs at an average power of 650 mW (measured as described below). For this study, we used a fixed wavelength of 750 nm.

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The laser was coupled into two different imaging systems: (i) a multiphoton tomograph (DermaInspect, JenLab GmbH, Germany) for skin imaging and (ii) a galvoscanning microscope (FemtoCut, JenLabGmbH, Germany) for imaging and nanoprocessing. The DermaInspect system is a clinically approved multiphoton autofluorescence tomograph. It is described in detail elsewhere. Key features relevant to this work are (see Fig. 1) a galvoscanning unit (GSI Lumonics, Inc., USA), a piezodriven (Piezosystems Jena GmbH, Germany) 40× focusing optics with numerical aperture (NA) 1.3 and a photomultiplier tube (PMT) (H5773P, Hamamatsu Photonics K.K., Japan). The system was further equipped with a 700 nm shortpass filter (AHF Analysetechnik AG, Germany) to prevent scattered or reflected laser light onto the detector. Mean laser powers were measured with a standard power meter (FieldMate, Coherent Inc. USA, with PM3 sensor head, maximum power of 2 W); the focusing optics of the tomograph as well as the objective of the scanning microscope were used without oil for pulse power measurements. A maximum power output of 88 mW after transmission through the tomograph could be realized with this laser source corresponding to a pulse energy of 0.09 nJ. Scheme and photograph of the galvoscanning microscope are depicted in Fig. 2. The instrument is based on a standard inverted fluorescence microscope (Zeiss Axiovert, Carl Zeiss AG, Germany) with a laser-scanning module designed for femtosecond laser manipulation (JenLab GmbH, Germany) attached to the side port. A 100× (oil) focusing objective with a numerical aperture of 1.3 (Carl Zeiss AG, Germany) has been employed. A maximum average power output of 130 mW with the gigahertz laser source was achieved.

B. Samples and imaging conditions

Green-fluorescent nanobeads with a diameter of 0.2 μm adhering to a cover slide were used to determine the point spread function (PSF) and for general alignment, 6 μm fluorescent microbeads (both Polysciences, Inc., USA) were employed. All pulse widths were determined with the auto-correlator “MINI” (APE GmbH, Germany), which features an external semiconductor diode for measurement of the laser pulse width in the focus of the high NA optics after passing through the laser systems. As biological samples, an Elodea densa plant tissue consisting of two cell layers and an excised human skin from frozen storage were employed. Studies on the healthy human skin were carried out on the left forearm of a female volunteer. All samples were imaged with multiple scan rates between 4.4 and 13.4 s/frame. Human metaphase chromosomes prepared from peripheral blood by standard methods were placed into a sterile cell chamber (MiniCeM, JenLab GmbH, Germany) and left to dry. One group of chromosomes was labeled with a 5% Giemsa (Merck KgaA, Germany) solution. All chromosome samples were laser processed with the laser-scanning microscope by single line scans and single point illumination at a scan rate of 17 ms/line (512 pixels, corresponding to 20 μm). The processing parameters resulted in a beam dwell time of 51 μs/pixel, thus 51 μs × 1 GHz ≈ 51 000 pulses with a peak power of 0.4 kW. Per single point, the chromosomes were exposed to an accumulated light energy of 0.1 nJ × 51 000 pulses = 5 μJ. For analysis of the laser-processed chromosomes, we employed an atomic force microscope (Topometrix, Santa Clara, USA). The samples were investigated in contact mode with 8 lines/s, creating a 512 × 512 pixel image with a physical size of 16 × 16 μm².

C. Data analysis

The contrast and signal-to-noise ratio (SNR) were calculated by comparing the medium intensities of two 100 × 100 pixel sections of an image showing fluorescent structures (yielding \( I_{\text{max}} \)) and background areas (yielding \( I_{\text{min}} \)), respectively,

\[
\text{contrast} = \frac{I_{\text{max}} - I_{\text{min}}}{I_{\text{max}} + I_{\text{min}}}
\]
$$\text{SNR} = \frac{I_{\text{max}}}{I_{\text{min}}}$$

III. RESULTS

A. Multiphoton tomography of human skin

The resolution of the tomograph was characterized first by determination of the PSF. Interestingly, we observed a laser beam induced displacement of nano- and microbeads when immersed in aqueous solution, similar to optical tweezers. Therefore, the PSF was determined on nanobeads in air (dried on a microscope slide). A lateral resolution of 0.4 μm [full width at half maximum (FWHM)] was determined, while the axial resolution reached 3 μm (FWHM). The measured maximum mean power after transmission of 88 mW corresponds to a transmittance of about 13%. The pulse width of the laser at the entrance of the system was 61 fs and was considerably stretched by the different optical elements to 310 fs. This resulted in a maximum output peak intensity of about 55 GW/cm² and pulse energy of about 0.1 nJ.

The imaging performance on biological tissue of the system with the gigahertz laser was tested with commonly used leaves of *Elodea densa* by generating multiphoton images to check the image quality prior to skin measurements. We were able to induce the multiphoton autofluorescence of the chloroplasts and cell structures. Subsequent scans of the sample revealed no visible changes in the image, even with full laser power.

We performed tomography of the skin of a forearm. Images at different tissue depths \( z \) (with an increment of \( dz = 5 \mu m \)) were recorded (Fig. 4). Compared to earlier results with megahertz laser systems, we observed the same features in the different layers of the skin at a similar image quality (contrast and SNR). \(^4\) Starting at the surface in the stratum corneum (\( z=0–20 \mu m \)) the typical hexagonal shaped cell structures of the stratum corneum (\( z=10 \mu m \)) were clearly identified. Regular horizontal line structures in the upper epidermis arise from motion artifacts, in particular, the volunteer’s pulse beat. In deeper areas the living cells in the stratum spinosum (keratinocytes) are visible. With increasing imaging depth the cross section of the cells gets smaller until the stratum basale was reached. The junction epidermis/dermis indicated by the presence of papillae started at a typical value of about 80 μm. It was possible to obtain multiphoton autofluorescence images throughout the whole epidermal layer until an imaging depth of \( z=100 \mu m \).

Figure 5 demonstrates a high-resolution autofluorescence image of the keratinocytes in the stratum spinosum showing the known composition of autofluorescence images of keratinocytes. In accordance with standard multiphoton imaging, the cell nuclei did not exhibit an autofluorescence signal in the visible spectral range.

B. Nanosurgery

On unlabeled specimens no chromosome dissection was achieved with the described configuration (gigahertz laser system with scanning microscope). Apparently the power density was insufficient to induce photodisruption. By staining with the chromophore blend Giemsa, the threshold for optical breakdown was significantly reduced. In this case we were able to process the chromosomes to realize incisions and to drill holes with dimensions in the submicron range.

Optical images of the chromosomes before and after laser irradiation are depicted in Fig. 6; the cutting lines are clearly visible. The effects of laser processing were analyzed...
by measuring the surface morphology and size laser pro-
cessed features with atomic force microscopy, see Fig. 7. The
measurements revealed cut sizes ranging from
250 to 300 nm and holes with diameters of 700 nm.

IV. DISCUSSION

A preliminary test of the image performance was real-
ized with a simple cell sample from plant tissue, *Elodea*
leaves. A clear contrast in the range of 0.7–0.8 (±0.05) and a
SNR of 10 (±0.5) show a good performance of the tested
configuration. As mentioned, consecutive images of the
sample configuration remained unaltered, thus showing no
evidence of instantaneous degradation of the sample fea-
tures.

In imaging human skin, we observed morphological
structures of the different skin layers as already observed in
experiments with megahertz laser sources by König and
Riemann.4 In accordance to these former examinations, the
fluorescence signal arises from the same area in the different
cell layers. Therefore, in the case of gigahertz lasers the ma-

or fluorescence component responsible for image formation
found in the stratum corneum is keratin produced in the ke-
ratinocytes. In the stratum spinosum, the reduced coenzyme
NAD(P)H mainly located in the organelles contributes the
most to the autofluorescence signal. Single organelles in the
cytoplasm of the cells were identified in accordance to the
earlier experiments.

For quantitative analysis, the SNR and contrast were
computed according to Eqs. (1) and (2) and compared to the
above mentioned earlier studies with a megahertz laser
source. In the stratum corneum, the SNR is in the range of
5–7 (±2) with a contrast of 0.6–0.7 (±0.05) for the system
employing the gigahertz laser, while typical values with the
megahertz systems are 5–8 (±2) and 0.6–0.7 (±0.05) for
SNR and contrast, respectively. In the stratum spinosum, the
SNR drops to 1.5–3 (±0.5) and the contrast to 0.12–0.3
(±0.03) with the gigahertz system, while typical values with
the megahertz system are 2–3 (±0.5) and 0.1–0.4 (±0.05) for
SNR and contrast, respectively.

In multiphoton microscopy and tomography, the conven-
tionally used megahertz-Ti:sapphire lasers are driven at av-
average powers of 10–40 mW, resulting in pulse energies of
0.2–0.5 nJ.4,20 In comparison with these standard systems,
the gigahertz laser employed in this work had to be operated
at an average power of 88 mW to obtain similar fluorescence
intensities. With this unusual high laser powers we realized
multiphoton fluorescence images with comparable SNR and
contrast values. However, this rather high average power ef-
effectively resulted in pulse energies still one order of magni-
tude lower than for megahertz laser sources.

Typical relaxation times of the excited states of fluores-
cent molecules found in biological tissue are in the region of
a few nanoseconds.4 The time window between two subse-
quent laser pulses is 12.5 ns for a typical 80 MHz laser sys-
tem, whereas it is only 1 ns for the 1 GHz system and there-
fore shorter or equal to the molecule relaxation times.
Therefore, the probability of excited molecules absorbing en-
ergy from a subsequent laser pulse rises and excited state
absorption is more likely to occur. The resulting energy
buildup is likely to result in molecule ionization and mol-
ecule dissociation. However, unaltered images were obtained
in consecutive scans of the same areas, thus revealing no
instantaneous visible degradation.

In contrast, destructive effects can be induced under es-
pecially used conditions, in particular, in stained biologi-
cal samples where the threshold for optical breakdown is
reduced. We demonstrated multiphoton ablation effects in
Giemsa stained human chromosomes by single point illumi-
nation and line scanning. In earlier works10 König et al. re-
ported an ablation threshold of about 1.6 TW/cm² for the
nanodissection of unlabeled chromosomes using a Ti:sap-
phire laser source with a pulse duration of 170 fs, a repeti-
tion rate of 80 MHz, and an average power of 40 mW, re-
sulting in a pulse energy of about 0.5 nJ. For Giemsa-labeled
chromosomes the ablation threshold was reduced by a factor
of 2.7. The maximum power density obtained with the giga-
hertz oscillator is about 55 GW/cm² with the galvoscaning
microscope and therefore not sufficient to process unlabeled
chromosomes.
By labeling the chromosomes, the ionization threshold of the targets is reduced so that the effect of excited state absorption is supposed to be able to generate the required energies for cutting or drilling of the chromosome material. On the one hand, this shows that the gigahertz laser can be used for the nanodissection of chromosomes and potentially other biological materials. On the other hand, it emphasizes the observations made with gigahertz multiphoton microscopy that the damage potential of on single pulse as well as the train of pulses with short temporal distance is not sufficient to induce damage to the specimen under examination. Rather, the consecutive pulses add to compensate the single pulse energies and add up to produce proper image qualities.

V. CONCLUSIONS

It was shown that gigahertz laser sources can successfully be used in multiphoton microscopy and nanomedicine. The achieved image quality was as good as that obtained with standard megahertz laser sources and produced the same features. Nanodissection could be demonstrated only on stained chromosomes. Since the time window between two pulses is in the range of the autofluorescence decay lifetimes of the target molecules, different physical effects compared to standard megahertz multiphoton microscopy have to be considered. Gigahertz femtosecond oscillators have the potential to become interesting multiphoton tools for high-resolution diagnostics and nanoprocessing of a variety of materials. Further systems have to be optimized regarding transmission and pulse broadening to be able to increase the transient laser intensities at the target of interest. As the development of beam scanners and photomultipliers advances to faster systems, gigahertz laser systems offer the additional advantage of an increased number of pulses during the pixel dwell time. This results in an increased fluorescence of a certain pixel in the obtained image.