Tunable, Low Repetition Rate, Femtosecond Pulse Ti:Sapphire Laser for *in vivo* Nonlinear Microscopy

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Multiphoton transitions of intracellular fluorophores used in microscopy can be efficiently excited in the 700-1200 nm spectral region, which wavelengths penetrate deeper in tissues and are much less harmful for living specimen than direct UV illumination in single-photon fluorescent microscopy. Photochemical damage mechanisms, which can cause oxidative stress or direct DNA damage, only can occur in the focus of the objective lens, where multiphoton absorption takes place. Single-photon absorption of the NIR radiation can cause photothermal damage, though this is not significant in most cells, where water is the major NIR absorber. However, in cells where other efficient NIR absorbers (e.g. melanin, hemoglobin, chlorophyll) are present, photothermal damage can be a problem. Since one of the most promising noninvasive diagnostic applications of multiphoton microscopy could be based on *in vivo* multiphoton imaging of skin tissue containing significant amount of melanin, photo-thermal damage effects should be taken care of. When the pulse repetition rate of the exciting laser radiation is not very low ($\nu >$ 5-10 MHz), the dominating effect responsible for temperature rise and thermal damage is the cumulative heating effect of the consecutive pulses, which is proportional to the time averaged power of the pulse train incident on the sample. Thus thermal damage can be mitigated by decreasing the average power, which can be achieved by the reduction of the pulse energy or by the reduction of the repetition rate. Since two-photon absorption rate, and thus the two-photon fluorescence signal are quadratic functions of the pulse energy and linear functions of the repetition rate, mitigating thermal damage by reducing the repetition rate is preferable.

In our invited talk, we present a new approach for reduction of the laser induced damage in specimen in nonlinear microscopy, which is specially important issue in *in vivo* diagnostics. Biochemical, genetic and thermal mechanical damage is likely to occur at even low power threshold applying two-photon excitation of biological specimen. We reduce the laser repetition rate by a factor of ~3.45 relative to a standard, 76 MHz, tunable, femtosecond pulse Ti:sapphire laser in order to minimize the laser average power on the sample, but to have the same signal to noise (S/N) ratio. We demonstrate this effect on flourescencent beads, where we measure the fluorescence intensity for a 76 MHz and our new, 22 MHz laser system. Having the same spectral bandwidth, average power and beam profile, a factor of 3.3 increase in the fluorescence intensity was observed. In addition, we observed more detailed picture of keratin distribution of murine dorsal skin by application of the low repetition rate laser. This data suggest that resolution of specific endogene chromophore is possibly influenced by intensity of laser impulses that can be exploitable for biomedeical research. The reduced repetition rate of our long cavity laser could be also advantageous in other 3D imaging applications such as fluorescence lifetime imaging (FLIM). We are convinced that new laser construction is ideal for *in vivo* nonlinear microscopy: it has a reduced repetition rate, broadly tunable (from 715 to 880 nm) ultrashort (~300 fs) pulses and can be pumped at moderate (~2.5W) pump powers. These features result in a higher signal to noise ratio, a lower photodegradation of the biological samples and a more cost efficient construction than in case of its 80 MHz predecessors.