

TWO-PHOTON MICROSCOPY IN THICK TISSUE: ABSORPTION EFFICIENCY AND PENETRATION DEPTH DEPENDENT ON EXCITATION PARAMETERS.

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Two-Photon microscopy is an optical sectioning fluorescence imaging technique which relies on the quasi-simultaneous absorption of two or more photons by a molecule. This process needs a very high density of photons, condition which can be given by the use of laser flash with some very short pulses, at high intensity, high frequency (MHz) and confinement within a small focal volume (fl). One of the most important optical limitation is that biological sample absorb strongly light, mostly at low wavelengths (UV-Visible light), limiting the penetration depth of excitation beam and propagation of fluorescence signal. Probability of multiphoton absorption is strongly depending on the excitation parameters (peak power, repetition rate, energy per pulse). Assuming that absorption efficiency of a fluorophore can be estimated by its kinetic of fluorescence extinction, we have measured on tumour cells the fluorescence extinction rate of nucleus stained with Hoechst (10 images every 6s525ms in XZT mode) under different excitation conditions (pulse energy from 188 pJ to 75 nJ) with a SP2-AOBS Leica Microsystems CLSM; 400Hz, zoom 5.99, 400-500 nm channel detection) equipped with a HCPLApo20x/0.7NA objective.

First, an Electro Optical Modulator (EOM, KD*P crystal, 90.1% transmission, 1/254 extinction ratio at 789 nm) was used to modulate the average power (from 30 to 193 mW) and energy per pulse (from 188 pJ to 2,5 nJ) delivered by a femtosecond laser source with a repetition rate of around 76 MHz at 800 nm (Mira 900F, 8W at 532 nm, pulsewidth 100 fs, Coherent Inc). An additional module (Cavity Dumper, Coherent Inc) had been coupled to reduce the repetition rate of the pulse (division ratio from 30 to 120) and concentrate the energy per pulse (respectively from 39nJ to 75 nJ). The analysis of the photobleaching kinetic rate of nucleus fluorescence for different condition of excitation (EOM or Cavity Dumper) have shown variation in term of efficiency of pulse, confinement (excitation/fluorescence) and depth penetration (from the tumour surface to 116 μm depth).

For the experiments with EOM, peak power was increased with effective average power (from 30 to 193 mW) delivered by femtoseconde laser, showing a deeper penetration (at 76 MHz about 86 μm with 394 pJ and 112 μm at 2.5 nJ) and a reduced kinetic rate of bleaching (from 5.2x10⁻³ s⁻¹ to 3.3 10⁻³ s⁻¹) in relation to absorption probability. When Cavity Dumper was used to concentrate temporally energy (effective average power from 70 mW to 30 mW), repetition rate was decreased (from 1,8MHz to 452 kHz) when peak power (from 257 KW to 478 KW) and pulse energy (from 39 nJ to 75 nJ) were increased. The excitation efficiency per pulse was respectively in relation with the energy of this pulse as shown by the kinetic rate of bleaching (from 13.2x10⁻³ s⁻¹ to 9.4 10⁻³ s⁻¹). The fluorescence distribution was also confined nearest the surface (about 37 μm at 1.8 MHz with 39 nJ and 66 μm at 417 kHz with 71 nJ). To conclude, for similar average power, the using of Cavity Dumper showed a higher excitation efficiency per pulse (probability>1 and destructive saturation), a higher confinement for fluorescence emitted but restricted to the surface. However, we have also showed by Multiphoton-Flim that a reduced repetition rate given by cavity dumper was a great advantage for very long lifetime values (about 1500 ns at DR120).