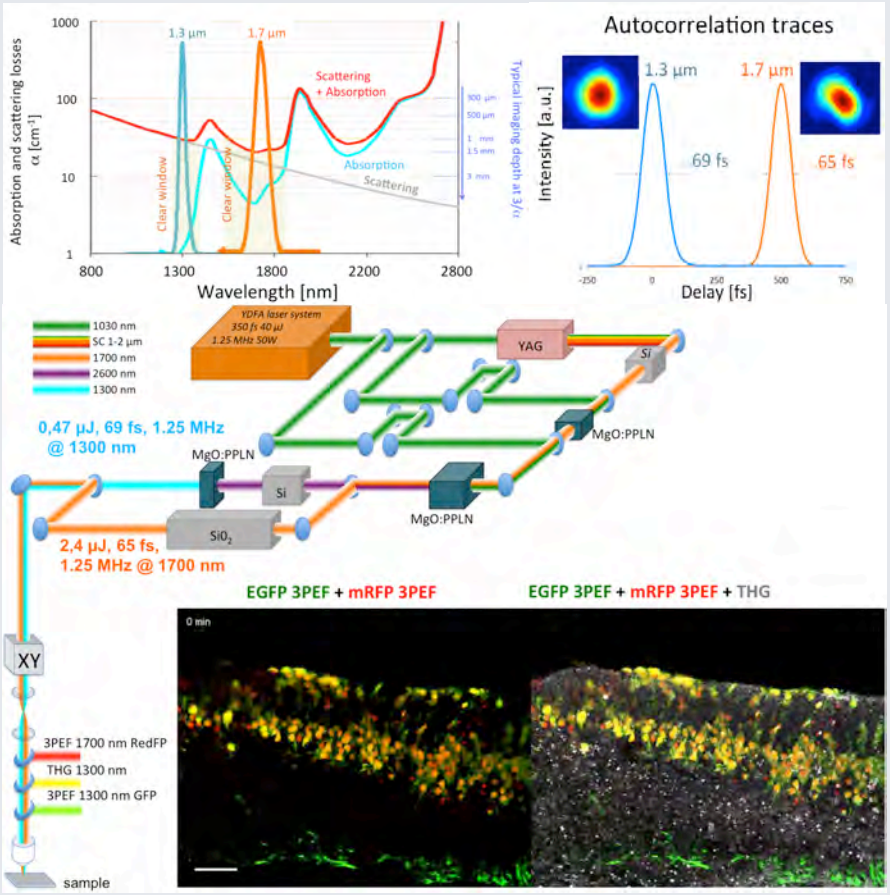
First demonstration of dual-color 3-photon non-linear microscopy

F. Druon, K. Guesmi, K. Jurkus, P. Rigaud, M. Hanna, P. Georges (LCF), E. Beaurepaire, L. Abdeladim, P. Mahou, W. Supatto (LOB), S. Tozer, J. Livet (Institut de la Vision).

One of the most challenging topics in optical microscopy consists in imaging deep inside biological tissues with high resolution. In addition, there is an ongoing trend toward the development of microscopes enabling the monitoring of several parameters on simultaneous independent channels. Since its introduction in the 90s, multi-photon imaging has made tremendous progress and has become the gold standard for deep/live fluorescence microscopy of biological tissues. This approach currently delivers subcellular resolution imaging in intact tissues at depths of a few hundreds of micrometers. However, the imaging depth remains a crucial limitation in heterogeneous and scattering tissues such as the brain and nervous tissues. To overcome this limitation and access to deeper areas, one very promising approach consists in using three-photon instead of two-photon excitation, while shifting the excitation to the SWIR (Short-Wavelength InfraRed) range. Indeed, scattering decreases with increasing wavelength, following a typical $1/\lambda^3$ law. However water absorption should be avoided to prevent tissue from heating; so 1300 and 1700 nm were recently shown (C. Xu et al, Cornell Univ., NY) to be optimal spectral exitation regions, offering the best compromise in terms of minimal scattering and absorption (fig). Efficient three-photon microscopy however requires MHz pulse trains of µJ-range pulses with sub-100fs durations. In 2017, we introduced and developped a novel laser source exceptionally suited for this pupose, providing 70 fs pulses at a repetition rate of 1.25 MHz and with energies in the µJ range. This new source is based on a robust OPCPA (optical parametric chirped pulse amplifier) injected by a high-power Yb-fiber system. One unique feature of our innovative design is that it emits simultaneously two beams at the two optimal 1.3 and 1.7 µm wavelengths. These wavelengths are suitable for exciting respectively GFP and RFP, two of the most widely used biological labels. We used our source to image dual-labeled chick spinal cords, and therefore achieve the first demonstration of dual-color 3PE microscopy, along with simultaneous detection of third-harmonic generation signals on a third channel (see figure).



(UP) (left) Optimal transparency spectral ranges for 3-P microscopy in biologically tissues such as brain. The blue axis represents the typical related maximum imaging depth. The blue and orange curves at 1.3 and 1.7 µm correspond to the measured spectra from aour laser source. (right) Corresponding autocorrelation traces and beam profiles at maximum energy. (DOWN) experimental setup and example of multimodal image obtained with our dual-1.3-and-1.7-µm source and example of images of developing chick embryo spinal cord tissue expressing cytoplasmic GFP labeling and nuclear RFP labeling (scale bar, 50 µm).

Besides providing innovative multicolor 3P excitation, our laser design outperforms alternative available sources in each 1300-and-1700-nm band. We also demonstrate the suitability of our dual-source for simultaneous in-depth high-quality multicolor 3P imaging of GFP-RFP labeled tissue along with label-free third-harmonic generation. This work is therefore the first demonstration of dual-color 3-photon imaging based on fluorescent proteins such as images given in fig. 1.

Guesmi, Abdeladim, et al, *Dual-color deep-tissue three-photon microscopy with a multiband infrared laser* (2017)

Patent application 1755167 (filed June 9th 2017): *Source laser ultrabreve pour l'excitation multiphotonique multicouleurs dans l'infrarouge moyen*

Résultats obtenus dans le cadre du projet DEEPIM financé par le thème émergence du LabEx PALM et porté par Frédéric Druon (LCF, IOGS) et Emmanuel Beaurepaire (LOB).