Deep Dive in Multiphoton Imaging

Multiphoton Imaging Enters New Areas of Bio-Science

In the past decade, Multiphoton Excited Fluorescence (MPEF) has firmly established itself as a major imaging technique for biological sciences. MPEF enables fast (video rate and beyond) 3D *in vivo* imaging of tissue and in some cases entire organisms. The technique relies on high peak power femtosecond pulses generated in the red and near infrared (NIR), thus allowing deep penetration (>1 mm) and sustained continuous imaging over long periods of time by limiting issues such as sample photo-toxicity, heating and damage.

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Cutting Edge Ultrafast Lasers for *In Vivo* Bio-Imaging

New ultrafast laser technology has been developed to create laser sources that can cover the entire relative transparency window of live tissue (700-1300 nm). Spectral tuning is fully automated and seamless, allowing access to any wavelength in a few seconds. The latest lasers feature dispersion pre-compensation, to counter the dispersive effects of transmissive optics on the laser path to the sample (that is, objectives, modulators and attenuators, combiners and in some cases the biological sample of interest itself). Cutting edge sources typically include multiple beams at various wavelengths to enable simultaneous excitation of various modalities such as the autofluorescence of endogenous elements naturally present in tissue (e.g. NADPH, flavin, elastin, lipofuscin) and the most commonly used fluorophores (e.g. GFP, YFP, mCherry).

Label free imaging modalities have also become essential elements of the biologist imaging tool box. Second and third harmonic generation (SHG & THG) imaging exploits the symmetry of structural proteins such as collagen (for SHG) and the interface of cell membranes (for THG). These structures can generate a coherent light response to nonlinear excitation in the NIR.

Furthermore, Coherent Anti-Stokes Raman Scattering (CARS) as well as Stimulated Raman Scattering (SRS) are label free techniques that take full advantage of the most advanced ultrafast laser sources. Such modalities can reveal endogenous molecules, for instance lipids. Technique refinements such as hyperspectral and broadband CARS are extending the use of ultrafast lasers in Raman imaging to the important fingerprint region, thus allowing the selective identification and imaging of chemical structures exhibiting specific Raman signatures [1].

Going Deeper, beyond Ti: Sapphire Technology

Traditionally, Ti:Sapphire laser systems covering roughly the short half of the transparency window (typically 700-1000 nm) have been the MPEF industry standard. To extend the spectral reach of Ti:Sapphire technology, some scientists have used optical parametric oscillators (OPO's) pumped by the fundamental wavelength of Ti:Sapphire. Ti:Sapphire pumped OPO optical setups typically suffer from a large footprint and potential instabilities inherent to the "2 box"

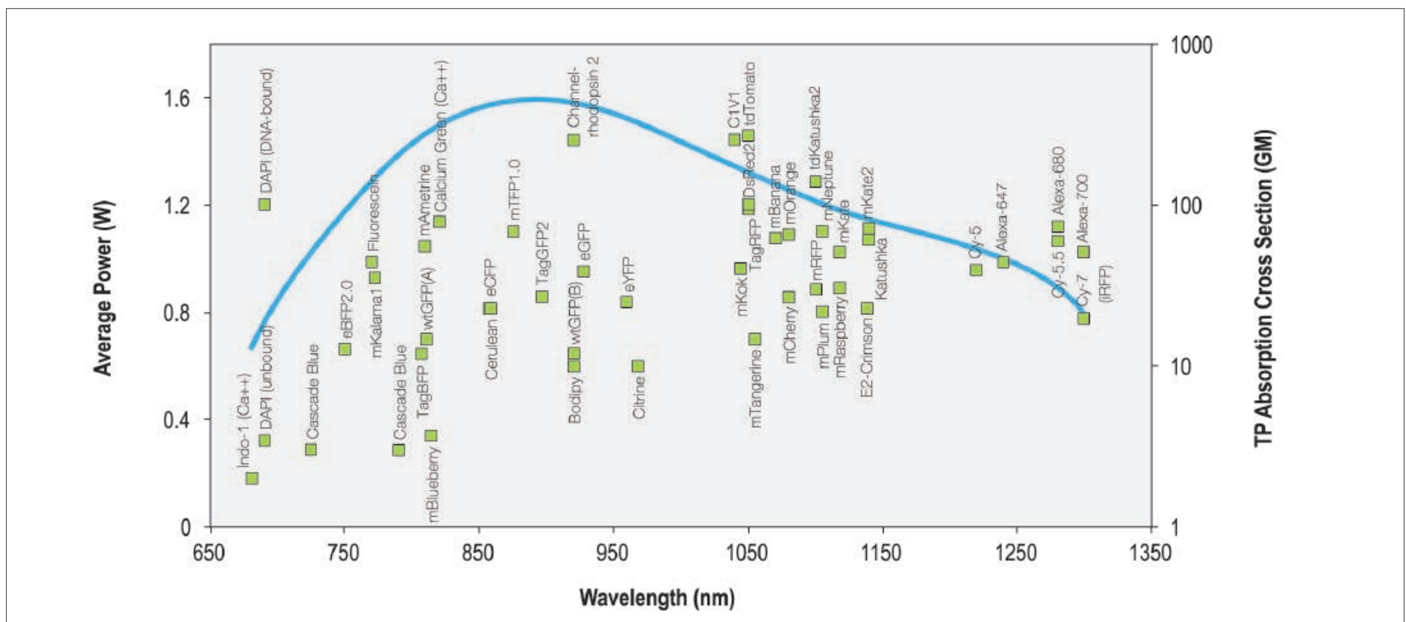


Fig. 1: Non exhaustive list of commonly used 2 photon fluorescent probes, shown as green squares as a function of peak two photon (TP) excitation wavelength (horizontal axis) and TP absorption cross section (secondary vertical axis). The average power tuning curve of InSight DS+ is shown in blue for reference [2, 3, 4].

approach. Moreover they provide limited peak power in the important 1–1.1 µm spectral region, where Ti:Sapphire reaches the edge of its gain and OPO output powers are weak.

The InSight DS+ platform constitutes an attractive alternative to Ti:Sapphire and its OPO extension. A true one box solution, this new technology delivers consistently high peak power across the entire transparency window including key wavelengths widely used by the MPEF community (920-930 nm for eGFP, 960 nm for eYFP). It is also an optimal tunable excitation source in the important spectral region from 1 µm to 1.3 µm, as illustrated in figure 1. This region is ideal for excitation of increasingly popular red shifted fluorescent proteins (RFP's) (e.g. mCherry, tdTomato, DsRed, E2-Crimson), as well as SHG/THG imaging.

Multiphoton Imaging, a Ubiquitous Technique in Bioscience

Nowadays MPEF imaging plays an important role in many areas of bioscience and beyond. For example, scientists utilize the technique in immunology, investigating graft and transplant rejection, as well as inflammatory response in small animals, for instance mice. In cancer research, MPEF imaging can be used to distinguish healthy from tumorous tissue, or track the spread of cancerous cells through the lymphatic system.

In the important field of optogenetics, ultrafast lasers are increasingly present, enabling the targeted photo-activation and silencing of individual neurons and groups of neurons in small animal brains. Research groups are starting to leverage new photo-activated channelrhodopsins (ChR) as well as genetically encoded calcium and voltage indicators (for instance RCaMP), optimized for 2-photon excitation in the NIR [5].

Outside biology, CARS imaging based on ultrafast lasers is used to detect and measure the distribution of specific compounds (for instance natural gas in geological samples) [6].

MPEF imaging techniques are routinely applied to small organisms such as zebrafish, C. Elegans, drosophila, and small marine creatures in embryonic or adult stage. Compared to larger animals, these organisms are relatively straight-

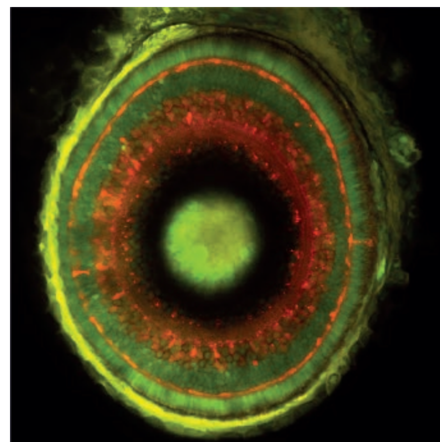
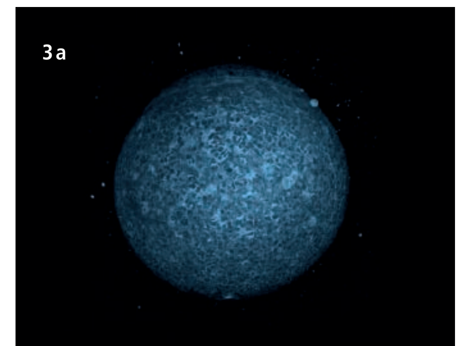
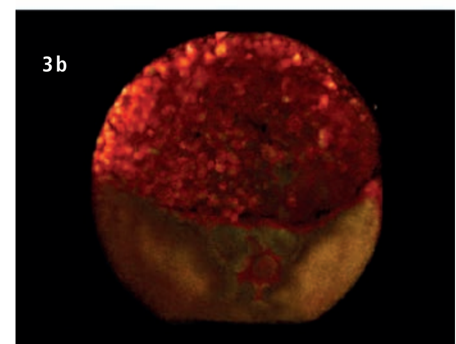


Fig 2: transgenic zebrafish embryo retina, image provided by Dr. Xana Almeida of the Harris Lab, Department of Physiology, Development and Neuroscience, University of Cambridge, UK





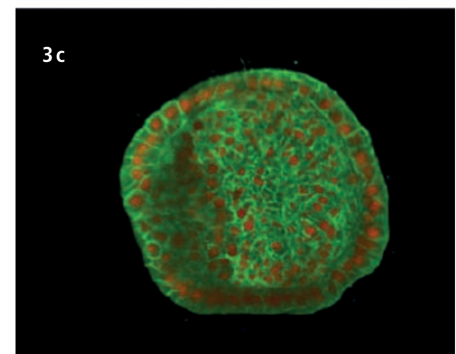


Fig 3: Zebrafish (a and b) and sea urchin; (c), images provided by Dr. Nadine Peyrieras, CNRS, Gyf-sur-Yvette, France.

forward, in some cases optically transparent and therefore often used as simplified models to answer a variety of anatomical and physiological questions. Characterization of the brain, retina, optical nerve, digestive system, vascular and lymphatic system is of particular interest.

Imaging Zebrafish Retina Using Dual Wavelength MPEF

Dr. Xana Almeida at the University of Cambridge, UK performs long term imaging of transgenic zebrafish retina. The retina is comprised of 5 neuronal cell types (photoreceptors, horizontal cells, bipolar cells, amacrine cells and retinal ganglion cells), which are responsible for processing and transmitting visual signals to the brain. Within the eye, these neurons are highly organized into separate layers that can be distinguished with simultaneous, dual wavelength MPEF imaging.

In figure 2, the green signal is obtained from excitation with the InSight DS+ tunable beam set at 927 nm, and results from the ubiquitous expression of H2B-GFP fusion protein driven by the Beta-actin promoter, labelling all nuclei.

The red signal is obtained from the fixed wavelength 1041 nm dual beam, and results from the expression of dsRed by the Ptf1-alpha promoter in 2 retinal cell types, Amacrine and Horizontal cells.

Both signals were obtained simultaneously, taking advantage of the two excitation beams from the laser system.

Observing Marine Organisms Embryonic Development *In Vivo*

Dr. Nadine Peyrieras and her team at the CNRS in Gyf-sur-Yvette, France investigates the embryonic development of small organisms such as zebrafish, sea urchin and dogfish. Figure 3 presents three specific examples of embryo studies, using various modalities (THG, MPEF imaging of various fluorophores).

Figure 3a shows a Zebrafish embryo (6 h post fertilization) using label free THG imaging with the tunable beam set to 1140 nm. In this case, THG provides an endogenous signal from cell membranes.

In figure 3b, a similar Zebrafish embryo (6 h post fertilization) is labelled with H2B:Venus for cell detection and tracking, and E2-Crimson, a far red protein requiring an excitation wavelength beyond 1100 nm. Both proteins were imaged with the tunable beam set to 1150 nm.

In figure 3c, *Sphaerechinus Granularis* (purple sea urchin) embryo is labelled with H2B-mCherry (red) and eGFP-RAS (green) revealing cell nuclei and membranes respectively. Both proteins were imaged with the tunable beam set to 980 nm

Conclusion

Next generation ultrafast lasers, such as are specifically developed for the multiphoton imaging community and enable new applications in bioscience and beyond. In particular, *in vivo* studies in immunology, embryonic development, cancer research and neuroscience all benefit from the new lasers' comprehensive and flexible spectral coverage, high peak power and dependable operation.

References

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