Fluence sp. z o.o.
ul. Kolejowa 5/7
01-217 Warsaw
Poland

Achieving Compact and Efficient Two-Photon Excitation Microscopy with the Halite Laser

Two-photon excitation microscopy (TPEF or 2PEF) is an essential tool in biological imaging. By exciting fluorescent markers using lower-energy photons and leveraging nonlinearities of the absorption process, TPEF offers a number of advantages(including reduced photobleaching, reduced scattering, and increased imaging depth).

Fluence Technology developed Halite, a series of best-in-class femtosecond lasers, to provide cost-effective solutions for the most demanding TPEF applications.

The Advantages of Two-Photon Excitation Microscopy

Since its demonstration in the 1990s, TPEF has developed into a powerful and widely-used biological imaging technique.[1]

The basis of all fluorescence microscopy techniques is fluorescent markers (known as fluorophores), which can be tailored to bind to specific sites and structures in complex biological samples and live tissues, such as individual cells of a genetically defined type. Moreover, fluorescent markers reveal high-contrast morphology, structures of samples, and tissues imaged via two-photon microscopes by emitting visible light.

To emit visible light, fluorophores must first be excited. In conventional microscopy, each chromophore is excited by a photon with higher energy (shorter wavelength) than the emitted light. TPEF subverts this method by achieving the required excitation using **two** relatively low-energy (long wavelength) photons whose combined energy approximately matches the energy of the emitted photon. Excitation occurs when these two photons are absorbed simultaneously by a fluorophore, leaving a molecule at a higher energy level.

There are numerous advantages of exciting a fluorophore using lower-energy photons.[2] Since scattering increases with energy, two-photon techniques exhibit much lower scattering and as a result, offer increased imaging depth and suitability for *in-vivo* imaging.[3] In addition, the photobleaching of the sample is reduced. Also, as a benefit of a combination of nonlinearity of the process and laser scanning mechanisms, TPEF offers significantly higher resolution in thicker samples than standard fluorescence microscopy.

The exact mechanism of obtaining high resolution can be explained as follows. Two photons can only be absorbed when light intensity is high enough to exceed a threshold value. This means that the light at low intensities can be transmitted through the tissue until it reaches high intensity in the focal point of the laser beam. In effect, it is possible to select a point very precisely in 3D,

avoiding the signal noise from surrounding tissue. This allows to precisely image deeper layers of the tissue.

An example of 2PEF in-vivo imaging of coronal slice in mouse cortex is presented in Figure 1.

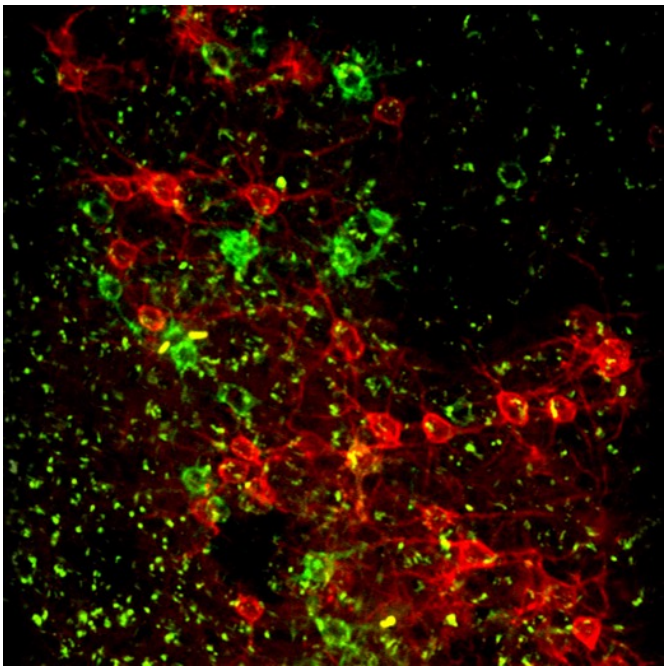

Figure 1 Coronal slice, mouse cortex. Histology of a mouse used for in vivo voltage imaging using Ace-mNeonGreen and Varnam (red) labeled neurons. Both fluorophores express in the cell membrane. Laser source: Fluence Halite (rep. rate 20 MHz, wavelength 1030 nm)

Overcoming Challenges in TPEM

The primary challenge in two-photon excitation microscopy is that the probability of simultaneous two-photon absorption is incredibly low. This means that extremely high incident photon densities are required to reliably produce fluorescence.

Subsequently, femtosecond lasers are key tools in TPEM. These devices emit a rapid series of very short and high-intensity laser pulses to produce the required photon densities without the high continuous power output of a continuous laser.

With a strong reliance on laser performance, the image quality obtained by two-photon techniques largely depends on laser parameters. Precise control of pulse duration and minimization of GDD (caused by optical elements in the laser path) are essential to achieve high-peak-power pulses that are as short as possible.

The Halite series of lasers from Fluence Technology offers a unique solution to two-photon imaging.[4]

Low repetition rate

Perhaps surprisingly, decreasing the rate at which a laser produces pulses (repetition rate) can *improve* performance. Suppose two lasers have the same average power but different repetition rates. In that case, the laser with the lower repetition rate (i.e., fewer pulses per second) must emit more energy per pulse. As a result, this means that lower repetition rates equate to higher photon densities, thus enabling higher quality imaging.

While competing systems typically offer pulse repetition rates in the region of 80 MHz, Halite lasers have a much lower 20 MHz repetition rate. This means that Halite lasers provide a **4x increase in pulse peak powe**r compared to an 80 MHz laser with the same nominal power. Figure 2 shows a comparison between TPEF performed with a 20 MHz laser and a 80 MHz laser at the same average power level. It is clear that 20 MHz brings a much better yield than a 80 MHz laser thanks to the higher pulse energy. In the 2 W power class, Halite femtosecond lasers offer the highest pulse energy available on the market at **up to 100 nJ per pulse**.

A lower repetition rate also means lower heat accumulation in the sample, which is critical for biological imaging.

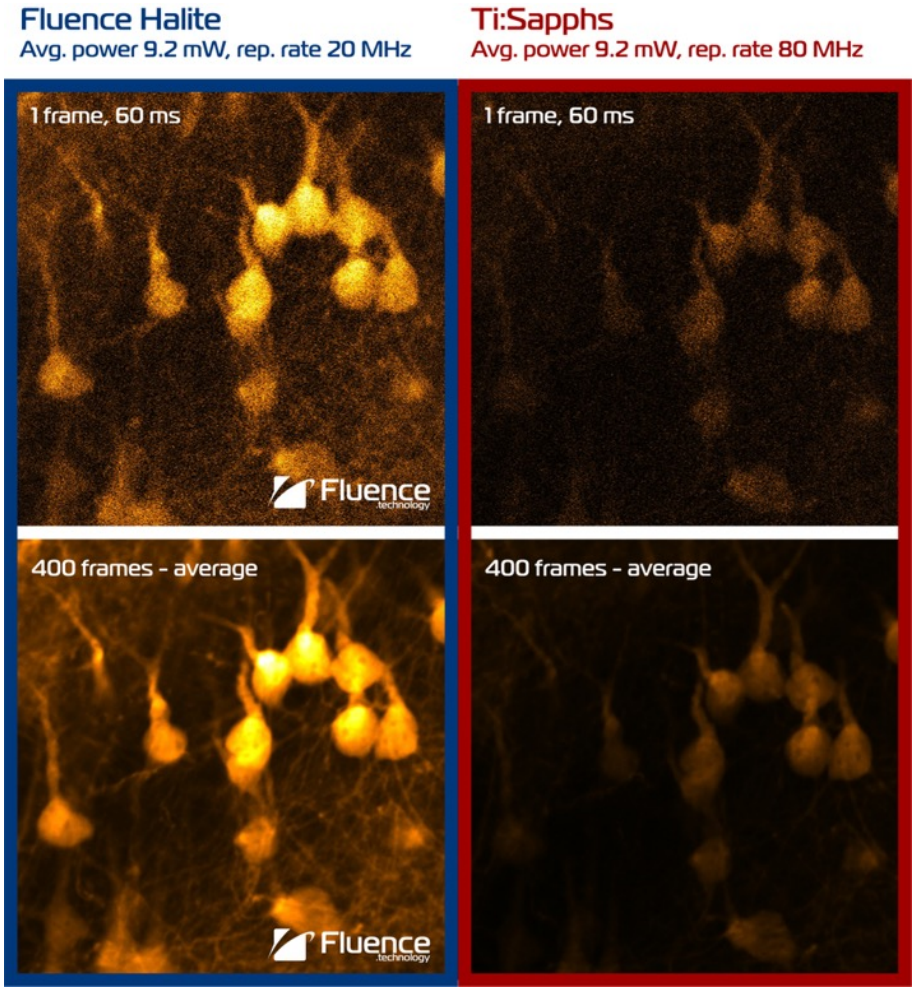

Figure 2 Laser source comparison. Fluence Halite (20 MHz) vs Ti:Sapphire laser (80 MHz). Structural 2-photon imaging at 1030 nm of pyramidal neurons and their dendrites. Fixed tissue, coronal slice, YFP-H mouse line.

All-Fiber Optics

Laser systems based on crystal technologies (such as Ti:Sapphire lasers) not only have very high capital costs (typically well above $150,000), but require additional maintenance and repair that can have a dramatic impact on lifetime costs. The robust all-fiber design of the Halite range eliminates bulk optics (including saturable absorbers like SESAM) from the optical train, preventing degradation of components and eradicating any requirement for future maintenance. No bulk optics means no misalignment, which prevents the need for time-consuming alignment and optimization.

The unique oscillator design of the Halite range ensures reliable performance in a variety of conditions: Fluence's femtosecond oscillator that Halite bases upon shows stable operation even in 40 g shock tests and remains stable in a vast range of temperatures. Halite lasers offer increased power stability and clean pulses with a typical pulse duration of 200 fs. At the same time, inbuilt GDD precompensation corrects for the group delay dispersion arising from the microscope's optics in a range from 10,000 fs^2 down to -100,000 fs^2. Each optical component between the laser source and the sample will usually stretch the pulse duration due to dispersion, leading to a drop in process efficiency. Automated GDD precompensation tuning may be beneficial for groups building microscope prototypes, where an optical train is unpredictable and constantly changing. It allows to simply add or subtract group delay dispersion to the pulse from the software level so that the optical train of the microscope compresses the pulse duration back to a minimum value at the sample. Therefore, automated GDD tuning also means that the user can freely tune with the pulse duration.

Compact Single-Box Design

With a compact all-in-one design, Halite lasers do not require any external cooling unit or controllers. It is the only laser in its class powered from a 24 V power adapter, with all electronics integrated into a single laser head. This makes Halite not only an elegant solution but also the smallest femtosecond laser for TPEF. Halite lasers are ultra-reliable light sources that can be easily integrated into any two-photon imaging system. Due to their compactness and stability, they can be readily incorporated into designs of the imaging systems operating beyond the laboratory settings, hospitals, R&D offices, etc.

Recently, Halite has been upgraded with even more power in a new generation of Halite 2, shown in figure 3. The new Halite 2 offers over 2 W of average power and pulse energy as high as > 100 nJ, obtained thanks to the reduced base repetition rate down to the level of 20 MHz. The fundamental wavelength of 1030 nm in Halite 2 is useful for TPEF and is also perfect for multi-photon excitation fluorescence microscopy MPEF, in particular three-photon microscopy (3PEF). Apart from its fundamental wavelength, Halite 2 can also come with the second harmonic option, generating 515 nm laser light at its output, which is ideal for applications like 2-Photon Polymerization (TPP, 2PP). To find out more about the Halite range and our other high-performance laser products, get in touch with a member of the Fluence Technology team today.

Figure 3. Thanks to its unique technology, Halite 2 is the smallest femtosecond laser with energy level >100 nJ at 20 MHz, and with no external controllers.

References and Further Reading

1. Denk, W., Strickler, J. & Webb, W. Two-photon laser scanning fluorescence microscopy. *Science* **248**, 73–76 (1990).
2. Larson, A. M. Multiphoton microscopy. *Nature Photon* **5**, 1–1 (2011).
3. Helmchen, F., Denk, W. Deep tissue two-photon microscopy. Nat Methods 2, 932–940 (2005). https://doi.org/10.1038/nmeth818.
4. Halite: Single-box compact femtosecond laser - Fluence.technology Fluence.technology. *Fluence.technology* https://fluence.technology/products/halite/.