How Mode-Locked Lasers Affect the Past, Present, and Future of Two-Photon Microscopy

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Introduction

For over 100 years fluorescence microscopy has been utilized in biological sciences as a means for identifying the spatial distribution of molecules of interest in complex heterogeneous samples. The first fluorescence microscopes invented by Otto Heimstaedt and Heinrich Lehmann relied on ultraviolet light to induce autofluorescence [1]. Shortly after, in 1914 Stanislav Von Prowazek showed that fluorescent dyes could be bound to living cells [1] allowing the excitation and emission wavelengths to be engineered independently of the samples native properties.

These fluorescent tags, as they later became known, quickly became standard practice in fluorescence microscopy for many years because they allowed the excitation energy required to induce fluorescence to be greatly reduced. This, in turn, reduced photochemical degradation of the sample by moving from ultra-violet to visible excitation wavelengths, which was extremely advantageous for biological studies allowing for sample integrity to maintained especially for live samples. As a result, fluorescence microscopy has become one of the most widely utilized techniques in biological sciences.

For many years the advantages of fluorescence tags outweighed the disadvantages of the sample preparation requirements, but with the emergence of two-photon fluorescence microscopy in the 1990s [2], many of these same advantages can now be achieved without any sample pretreatment. As a result, two-photon microscopy, also referred to as multiphoton microscopy, has once again revolutionized the field of biological imaging allowing for the creation of high-resolution images of living cells. Even today, new uses are constantly being discovered.

In this article, we will explore how two-photon microscopy has evolved over the years and the integral role that the mode-locked laser has played in its evolution.

Historical Overview

The first prediction of multiphoton excitation was presented in Nobel laureate Maria Göppert-Mayer's dissertation in 1931, and then experimentally verified 30 years later by multiple research groups including Franken et al. and Kaiser & Garret. Even though the first mode-locked laser was demonstrated around the same time by Logan E. Hargrove, Richard L. Fork, and M.A. Pollack, it took another 30 years before Winfried Denk and his colleagues at Cornell University used one to build the first two-photon microscope. [2]

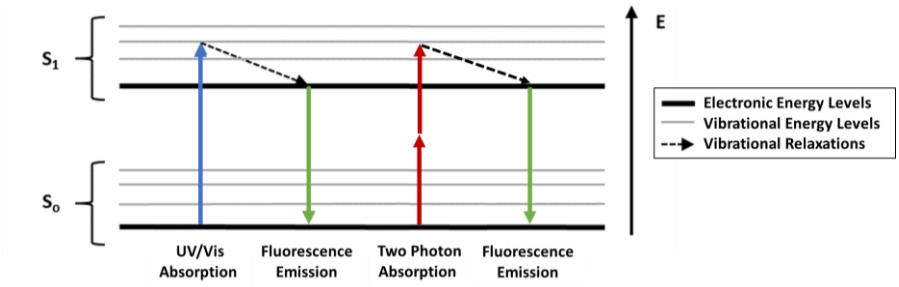

Figure 1. Jablanski diagram of traditional and two-photon fluorescence.

In order to fully understand the early technological challenges with this technique, and the important role the mode-locked laser has played in overcoming them, it is important to first have a fundamental understanding of the underlying nonlinear process at hand. As shown in Figure 1 above, two-photon fluoresce utilizes sum

frequency generation inside of the sample in order to induce a fluoresces excitation equivalent of a photon with twice the excitation energy.

The simplest way of understanding this nonlinear optical effect is by looking at the relationship between the polarization density (**P**), the materials susceptibility tensor (χ) which is related to the index of refraction of the marital, and the electric field of the incident laser (**E**). Sum frequency generation, also referred to as second harmonic generation, is what is known as a second-order nonlinear effect and obeys the following relationship,

$$\boldsymbol{P}^{(2)}(\boldsymbol{r},t) = \varepsilon_o \chi^{(2)} \boldsymbol{E}^2(\boldsymbol{r},t). \qquad \textit{Equation 1}$$

From Equation 1, it is clear that the effect is highly dependent on the magnitude of the electric field. Since the electric field density is dependent upon both the laser's spot size and the pulse duration, a tighter focus and a shorter pulse width will result in more efficient two-photon absorption.

In a diffraction limited microscope, the spot size at the sample is determined solely by the numerical aperture of the objective lens and the laser wavelength, that leaves the pulse duration as the only major variable in the system. For this reason, mode-locked lasers which produce ultra-short pulses (on the order of 100 fs) at high repetition rates have become the standard excitation source for two-photon microscopy. Moreover, the non-linear nature of the interaction ensures that multiphoton absorption only exists in the focal plane, outside of which the intensity drops too sharply for a non-linear interaction to occur. As excitation cannot occur outside of the focal plane, there is little to no out-of-focus light, resulting in clearer images.

In Winfried Denk's original work he utilized a mode-locked dye laser to produce approximately 100 fs pulses at 630 nm to excite a cluster of 9 μm fluorescent latex beads. While dye lasers may be suitable for demonstration in a university laboratory, they are far from commercializable. As a result the standard expiation source for two-photon microscopes quickly became the model-locked Ti:Sapphire laser. Aside from the obvious advantages of utilizing a solid state laser source, the Ti:Sapphire was also advantageous because it is widely tunable in the near infrared region, which causes minimal damage to samples and can penetrate much deeper into these samples compared to other commonly used visible wavelengths.

While Ti:Sapphire represented a huge improvement over other early mode-locked laser sources, it does have one major drawback. The peak lasing efficiency of Ti:Sapphire is around 780 nm while the majority of fluorescent proteins of interest to biologists have a two-photon absorption band primarily in the 900 nm to 1100 nm range, as shown in Figure 2 above. Additionally, it has been shown that there is a significant decrease in damage to living tissue by tuning the laser from 780 nm to 920 nm [2]. As a result, in most two-photon microscopes the laser is tuned to 920 nm where unfortunately Ti:Sapphire lasers are far less efficient.

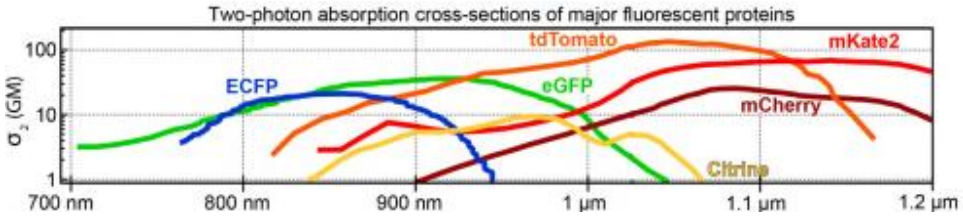

Figure 2: Two-photon absorption cross-section of select fluorescent proteins [3].

New Developments

In recent years there has been rapid developments in the field of mode-locked solid-state lasers, particularly mode-locked fiber lasers. Now there is a wide variety of lower cost, fit for purpose mode-locked lasers sources available on the market. Recently Spark Lasers (Bordeaux, France) developed one such laser source specifically tailored to the needs of two-photon microscopy applications. The Alcor laser series, shown in figure 3 below, is a mode-locked fiber laser, producing ultra-fast (<140 fs) pulses with 80 MHz pulse repletion rate at 920 nm and 1040 nm.

Figure 3: ALCOR-920 Mode-Locked Fiber Laser by Spark Lasers

The introduction of the Alcor laser series offers many advantages over the traditional Ti:Sapphire lasers used in most two-photon microscopes. In addition to the obvious technical advantages associated with working at 920nm, the Alcor laser series is lighter, more compact, air cooled, and less expensive. This allows for a simpler and cost-effective integration into microscopy systems.

The development of new mode-locked fiber lasers technologies, like the Alcor laser series, is allowing for the expanded adoption of the technique in biological science labs all around the world. This, in turn, is opening up new application areas, as more and more people are gaining access to the technology.

To find out how you can utilize the Alcor laser series or any other laser technologies in your lab or product visit www.rpmclasers.com or talk to one of our laser experts today by calling 1-636-272-7227.

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References

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