Improving laser standards for three-photon microscopy

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Introduction

Three-photon excitation microscopy has double-to-triple the penetration depth in biological tissue over two-photon imaging. The laser technology used for generating high-energy pulses used for three-photon excitation is relatively new and the repetition rates of the laser sources are typically low (1–2 MHz). Thus, pulse shape distortions and pulse-to-pulse variability can markedly impact image quality. We implemented state-of-the-art pulse measurements and developed new techniques for examining the performance of lasers used in three-photon microscopy. We then demonstrated how these techniques can be used to provide precise measurements of pulse shape, pulse energy and pulse-to-pulse intensity variability, all of which ultimately impact imaging.

Methods

We built diagnostic tools, e.g., a second harmonic generation frequency resolved optical gating (SHG-FROG) device, and a deep-memory diode imaging (DMDI) apparatus, to examine laser pulses from a 70W Spirit pump laser coupled to a non-collinear optical parametric amplifier (NOPA) set at 1300 nm central wavelength. Simulations and data analyses were performed in Python using custom scripts (see Ref [1]). Imaging of florescent samples was performed with a commercial multiphoton microscope (Investigator, Bruker Nano), which we further customized for three-photon imaging [2].

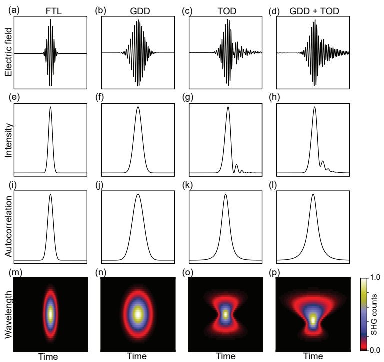
Laser pulse shape and intensity affect image brightness

There are many factors that influence the 3-photon excitation (3PE) signal photons per pulse and therefore quality of an image in three-photon microscopy, but some parameters are limited by the laser itself [3].

$$\text{3PE photons per pulse} = \frac{1}{3}\frac{g_p^{(3)}}{\tau^2}\phi C(\eta\sigma_3)n_0\frac{2\pi^2}{3\lambda_{3p}^3}\left(\frac{\lambda_{3p}}{\pi w_0}\right)^2\left(\frac{P_{3p}}{f}\right)^3$$

In this study we investigate the effects pulse shape (which is intrinsically tied to the coherence factor g_p), and the pulse duration τ. These parameters together with the energy of the laser pulses determine the peak intensity and therefore excitation efficiency in three-photon imaging. Beyond the excitation efficincy of each pulse, If any of these parameters vary on the scale of microseconds to milliseconds to seconds, the excitation efficiency will vary across the image producing artifacts that interfere resolving the specimen of interest.

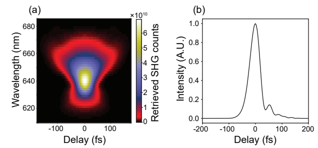
Pulse distortions induced by TOD can go undetected by autocorrelators



Due to their large bandwidth, laser sources used for three-photon microscopy acquire more of a distortion as they propagate through material than laser sources used for traditional two-photon microscopy. As a result of this, devices that are designed to compress laser pulses (compensate group delay dispersion - GDD) to the ultimate limit (fourier transform limit - FTL) fall short. Higher order distortions such as third order dispersion (TOD) remain, and devices such as autocorrelators cannot determine the extent of this distortion, which requires a spectral phase resolved diagnostic such as Frequency resolved optical gating (FROG).

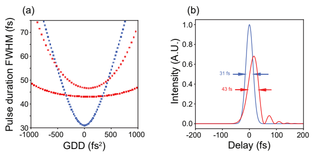
Measuring pulse shape and simulating its effect on image brightness

FROG measurements reveal TOD



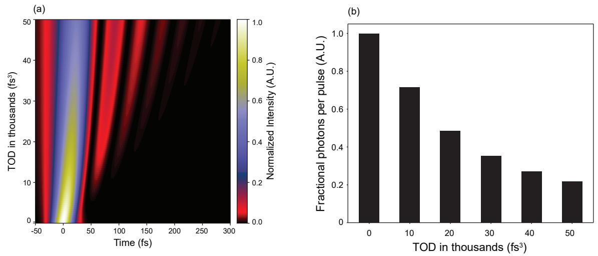
Laser pulses from our laser source were measured by SHG-FROG whose spectrogram (a) and pulse profile (b) show the presence of TOD and GDD. Qualitatively the asymmetric hourglass shape seen in (a) and beating structure in the tail of (b) indicate the presence of GDD and TOD, as seen in the previous panel. The GDD can be compensated with traditional pulse compressors such as prisms or gratings, but the TOD cannot, leading to efficiency losses.

TOD causes decreased peak intensity and incompressible pulses



The compression of laser pulses with our measured TOD value and pulse duration was simulated (a) to show that the pulses (1) cannot be compressed to their ultimate limit (FTL), and also that the estimation of pulse duration by using intensity autocorrelations deviate strongly from the actual pulse duration as determined by the FWHM of the laser intensity profile. When laser pulses are completely compressed (all GDD removed), the peak intensity of laser pulses falls by ~ 32% of the value it would be in the absence of TOD.

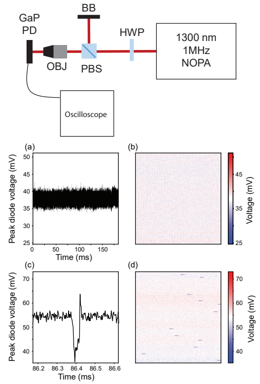
Larger TOD further exacerbates loss of peak intensity and image brightness



Simulations were performed with our laser pulses to determine the effect of a range of TOD values on pulse profile and therefore peak intensity. As TOD is increased, energy from the laser pulses is transported to the wings of the pulse profile as seen in (a). With more TOD, the peak intensity correspondingly drops leading to a decrease in the three-photon signal. The expected three-photon fractional signal photons per pulse was calculated based on the coherence factor and pulse duration of our aser pulses and shown to be less than half of what it would be if all TOD were removed. Thus TOD must be addressed to achieve the best signal brightness when imaging deep in the brain.

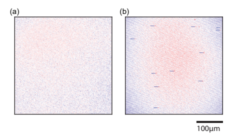
Diagnosing Pulse-to-Pulse Intensity Fluctuations

Deep memory diode imaging (DMDI) reveals intensity instabilities



To test the pulse-to-pulse intensity stability of the laser, we developed a technique called deep-memory diode imaging (DMDI). In DMDI, pulsed laser light is projected onto a photodiode and the photodiode signal is recorded by a high sampling rate oscilloscope with a large buffer to capture the pulse signal peaks at a high temporal resolution. To match the order of photo-absorption process in three-photon imaging, a tight-focusing element (e.g., a microscope objective) is used to focus the laser onto a photodiode with an appropriately chosen band-gap that disallows one- and two-photon excitation [e.g., GaP]. Like three-photon excited fluorescence, the photodiode signal recorded in this way is sensitive to peak pulse intensity and not just to total pulse energy. A high sampling rate and large buffer memory oscilloscope is used then to record high resolution signal peaks that are then reconstructed from a series of summed peak voltages into a 2D 'image.' Two sample diode images can be seen in (b) and (d), where (b) shows a relatively flat field ideal for imaging, and (d) has short streak-like artifacts that were not captured by pulse-to-pulse statistics like RMS.

Instabilities in pulse-to-pulse intensity can result in imaging artifacts



The microscope images shown in (a) and (b) correspond to the same laser conditions as in the previous section. Here, a fluorescent slide is imaged by our microscope to approximate a flat field brightness to better visualize the intensity variability due to the laser itself. As can clearly be seen, the streak-like artifacts that were measured by DMDI are also present in (b), and were not able to be identified by typical pulse-to-pulse stability metrics such as RMS.

Summary

Our diagnostics provide a means to determine the performance of multi-photon laser sources used in three-photon fluorescence microscopy applications. Using these methods, we found that:

1. The distortion of individual laser pulses caused by TOD limits the compression of laser pulses. Thus, signal brightness is reduced. Though not presented here, we have introduced table optics to minimize TOD.
2. Pulse-to-pulse variability can be readily detected and unless corrected, will degrade image quality.

Adopting our diagnostic standards have led to the laser manufacturer updating our system that has yielded stable pulses 24/7, which we still have for 18 continuous months of uninterrupted usage.

References

Liu, C.J., et al. Sci Rep 10, 16351 (2020)
Farinella, M.D., et al. Neurophotonics 8, 015009 (2021)
Wang, T., et al. Optica, 7, 947-960 (2020)

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