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Is laser repetition rate important for two-photon light sheet microscopy?

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Abstract: We demonstrate the thermal advantages of using low repetition rate, high peak power lasers for imaging in two-photon light sheet microscopy using a Bessel light beam. We compare the use of two ultrashort pulsed lasers in such an imaging system: a high repetition rate source operating at 80 MHz and a low repetition rate source operating at 1 MHz. The low repetition rate laser requires approximately one order of magnitude lower average power than the high repetition rate source to yield the same fluorescent signal. These lasers are used to image Zebrafish larvae and record their heart rates. The data show heart rate values 30% in excess of the ground truth baseline value when imaged with the high repetition rate source due to deleterious heating, whereas the low repetition rate source yields data only a few percent above this ground truth value.

1. Introduction

Multi-photon fluorescence microscopy, particularly in two-photon mode, is now established as the optical method of choice for deep imaging and *in vivo* studies. Multi-photon excitation with longer wavelengths of light reduces the effects of light scattering. When performed with exogenous fluorophores, depths in various forms of two-photon imaging (point scanning mode, wide field temporal focusing and in light sheet geometries) can exceed several scattering lengths, which corresponds to distances of up to one millimetre into tissue. Additionally, the nonlinear nature of multi-photon fluorescence processes confines excitation to a smaller volume than is the case for single-photon excitation. This may minimize photobleaching away from the focus within the biological sample while simultaneously reducing out-of-focus light that may be detrimental to the recorded signal [1].

Due to the requirements of two-photon excitation, an ultra-short pulsed laser of femtosecond duration is typically required. A central aspect for this form of imaging that has gained only limited attention to date is the repetition rate of the excitation laser: essentially this is a trade off between peak power and average power. The majority of fluorescent dyes exhibit lifetimes up to 10 nanoseconds, which in turn dictates that the typical repetition rate for the laser source should be below 100 MHz [2]. As a result of this, lasers close to this repetition rate, which are readily accessible (e.g. sources at 80 MHz), have been the dominant source of choice for two-photon microscopy. However, lasers with lower repetition rate, higher peak power and lower average power can be harnessed in experiments to retain the same emission signal while potentially reducing heating. To secure this advantage we should remain below any nonlinear photodamage threshold. This premise is particularly pertinent to the area of light sheet microscopy (LSM).

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LSM has gained exceptional attention based on its low phototoxicity and fast acquisition rates and promise of very long interrogation periods for biomedical studies [3,4]. In point-scanning geometry, laser pulses and scanning systems can be synchronised to deliver down to a single pulse per pixel achieving frame rates faster than 15 fps for a 1024×1024-pixel field-of-view. Due to the particular geometry of LSM, the requirement for high repetition rate for fast imaging is not as critical and a lower repetition rate laser may be used.

We describe the use of lower repetition rate lasers for LSM in two-photon mode. The aim of the study is not to optimize image quality but to show that, for equivalent fluorescence signal, the use of higher repetition rate (which is accompanied with the use of higher average power), has a deleterious effect on the sample. Our study provides clear biological evidence for the fact that the use of such a low repetition rate femtosecond laser source (1MHz), versus the ubiquitous high repetition rate laser source (80MHz) typically used in such studies, results in a substantially lower degree of sample heating. This is quantified by our direct observations of the heart rate of zebrafish which, for the same recorded signal, exhibit a 30% increase due to laser heating for the case of the high repetition rate source. This study concurs with separate work exploring the use of laser frequency for LSM imaging as a sensitive probe of linear and nonlinear photoperturbations in zebrafish [5]. Overall, we envisage that the observed effects of sample heating and its mitigation by the use of lower average power, lower repetition rate lasers will influence and guide laser choice for future studies in LSM.

2. Methods

A digitally scanned two-photon Bessel beam light sheet fluorescence microscope, similar to one we previously demonstrated in [6], was implemented as shown in Fig. 1. Bessel beams are part of the family of propagation invariant light fields which have been particularly useful in light sheet imaging to retain high resolution across a large field of view [7–9]. The Bessel beam in particular is very useful for both two- and three-photon based light sheet imaging [6,7]. The beam depth of field (DOF) with uniform intensity, and thus usable horizontal field of view (FOV), was 300μm. The microscope optical path was shared by two femtosecond lasers, a Coherent Chameleon Ultra II (80MHz Repetition Rate) laser and a Coherent Monaco-pumped Opera-F (1MHz Repetition Rate) laser. This setup was used to image the beating heart of different zebrafish (transgenic line Tg(cmlc2:EGFP)) over periods of 30 minutes.

Zebrafish are popular organisms in biophotonics studies, due to their transparent nature and regenerative properties. They lend themselves to studies in drug development as well as cardiovascular and neuroscience research. Furthermore, their heart rate is known to be susceptible to changes in temperature [10,11]. Zebrafish larvae were raised in 10mM HEPES buffered E3 medium at 28.5°C. For the imaging procedure, 5 days post-fertilisation larvae were anaesthetised in 0.5mM tricaine solution. Single-plane images of the zebrafish heart were acquired under brightfield illumination or through two-photon fluorescence in our light sheet system (see Fig. 2(a)) excited by either laser.

For each fish, the average power of each laser was set before the fluorescence recording of the initial time point to reach an equivalent recorded fluorescence signal: average power Opera-F = 33±4.7mW, Chameleon = 336±35mW (Fig. 2(b)). The laser pulse width at the image plane was measured with a CARPE autocorrelator (APE) and resulted in values of 217fs for the Chameleon laser and 300fs Opera-F laser. For two-photon excitation, the amplitude of the virtual light sheet was set so that only the heart of the zebrafish was directly illuminated. The parameters used are summarised in Table 1. As can be seen in the illustrative examples of Fig. 2(a), in these conditions the time taken for one complete period of the heart beat differs. Heart rate frequencies were extracted from the recordings using Fourier analysis of the mean intensity within the region of interest [12,13] (Fig. 2(c)). The uncertainty in the measurement of heart rate is given by the width of the peak in the Fourier spectrum, which is transform-limited with a HWHM of 5 bpm.

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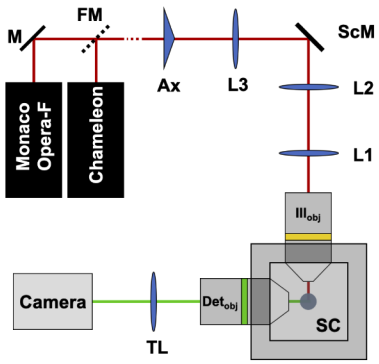

Fig. 1. Schematic illustration of the experiment system. M: Mirror (Thorlabs - BB1-E03), FM: Flip Mirror (Thorlabs - NX1N/M, BB1-E03), Ax: Axicon (Thorlabs - AX252-B), L1,L2,L3: Lens (Thorlabs - AC254-100-B-ML, AC254-050-B-ML, AC254-100-B-ML 100,50,100), ScM: Scanning Mirror (Thorlabs - GVS001), illObj: Illumination Objective (Nikon CFI Plan Fluor 10x 0.3 NA), DetObj: Detection Objective (Olympus UMPLFLN20XW 0.5NA), TL: Tube Lens (Thorlabs - AC254-200-A-ML), Camera (Hamamatsu Orca Flash 4.0 v2), SC: Custom made Sample Chamber, Emission filters: Laser2000 - FF01-520/60-25, SHPF-25C-790.

Table 1. Laser Parameters used in the Zebrafish Experiment.

	Chameleon	Opera-F
Average Power (AP)	336±35mW	33±4.7mW
Repetition Rate (RR)	80MHz	1MHz
Pulse Width (PW)	217fs	300fs
Pulse Energy = AP/RR	4.2nJ	33nJ
Peak Power = $AP/(RR * PW)$	19355W	110000W

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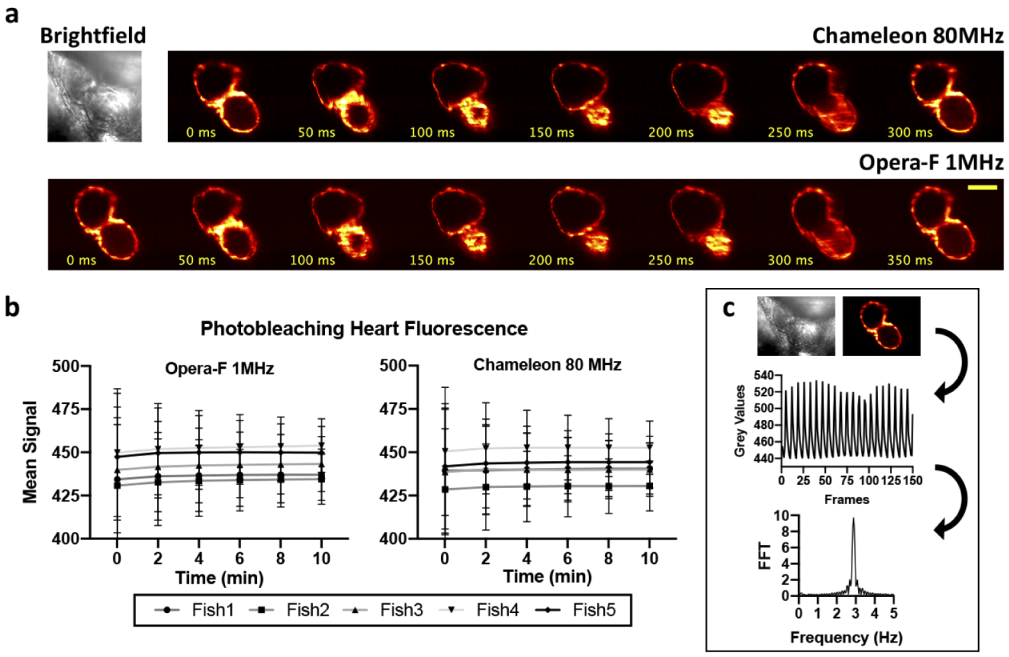

Fig. 2. **a** Example of brightfield (single frame) and two-photon Bessel light sheet fluorescence microscopy images of a zebrafish heart (Tg(cmlc2:EGFP)) beating cycle recorded at 20Hz with both Chameleon and Opera-F lasers at 920 nm. Scale bar = 30μm. **b** The zebrafish heart mean fluorescence signal recorded at 6 time points (150 frames at 20Hz each) every 2 minutes under constant laser illumination shows a similar low photobleaching rate for both the lasers. For each fish, the average power of Chameleon and Opera-F lasers was set at the beginning of the first time point to reach a similar fluorescence signal (average power: Opera-F = 33±4.7mW, Chameleon = 336±35mW). **c** Schematic pipeline used to extract the heart rate frequency information from brightfield and fluorescence stacks of images recorded: FFT and power spectral density (PSD) are calculated from the overtime signal variation and the frequency peak of the PSD is the beating rate.

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3. Results

We first examined the effect of the two lasers on fluorescence photobleaching. As shown in Fig. 2(b), for each zebrafish heart the mean fluorescence signal was recorded under constant laser illumination at six time points over a 600s time window. The data indicates that both lasers performed similarly to within 2% with a low photobleaching rate, independently of the repetition rate. We then used the Fourier analysis to extract the actual zebrafish heart rate from the brightfield and fluorescence recordings.

To measure the effects of the laser excitation on the zebrafish heart rate, single-plane time-series of 150 frames at 20Hz were recorded over a 30 minutes period for each fish: first under brightfield illumination to acquire the baseline (5 recordings every 2 minutes), then under continuous two-photon excitation at 920 nm either with Chameleon or Opera-F lasers (six recordings every two minutes; the lasers were run continuously, even when not recording), and finally under brightfield illumination again (Fig. 3(a)). The measurement was repeated so that the heart rate of each fish was monitored under illumination by each laser.

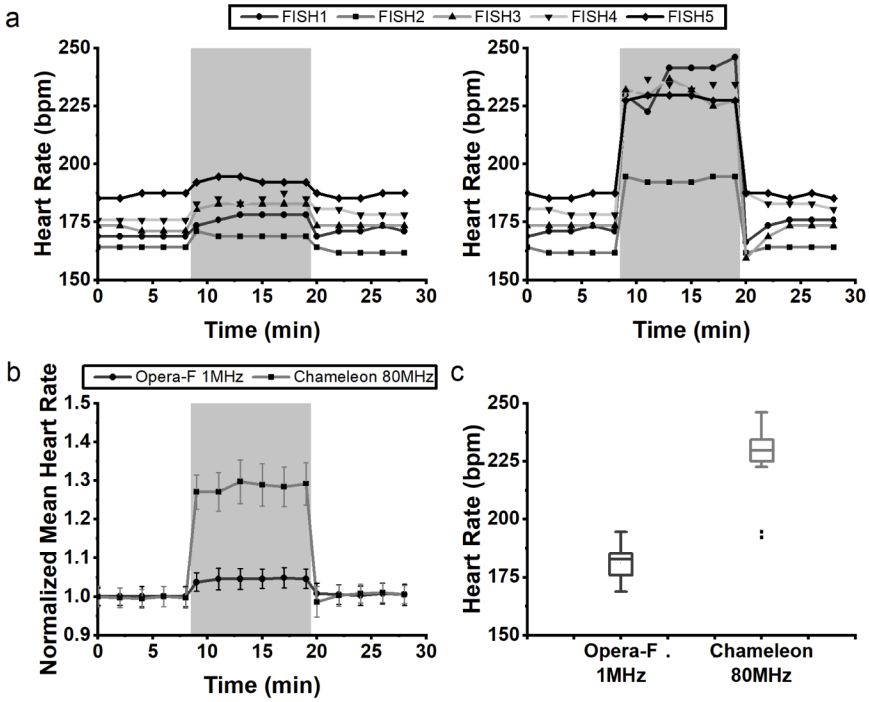

Fig. 3. **a** Raw heart rate of the zebrafish extracted from Fourier analysis, under conditions which result in comparable fluorescence signal for the same exposure time. White areas denote brightfield illumination, while grey-shaded regions show the heart rate under continuous two-photon Bessel light sheet illumination generated with (left) an Opera-F 1 MHz-repetition rate laser and (right) a Chameleon 80 MHz repetition rate laser. **b** Average heart rate under illumination by each laser (normalized to the value at zero-time) for the same data as shown in **a**. The increase in the average heart rate under light sheet illumination is notably larger for the high repetition rate, high average power laser. Error bars denote the standard error in the mean value of the heart rate. **c** Box plot of recorded heart rates during light sheet illumination by each laser, showing a significant difference in the heart rate observed under illumination by the two lasers.

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Figure 3 shows that the Chameleon laser, on average, increases the zebrafish heart rate by about 30%. On the contrary, the Opera-F laser has a substantially lower effect and the heart rate is, on average, just about 5% higher than the ground truth baseline value. We emphasize that the change in heart rate is significantly higher for illumination with the high repetition rate, high average power light source. Although the sample size is small, the zebrafish heart rate after 10 minutes of continuous illumination by the Chameleon laser (mean = 226 bpm, standard deviation = 19 bpm) is significantly larger than the heart rate after 10 minutes of continuous illumination by the Opera-F laser (mean = 181 bpm, standard deviation = 9 bpm). A paired-samples one-sided t-test gives $t(4) = -6.3, p = 0.0017$, while a paired-sample Wilcoxon signed rank test gives $Z = -1.9, p = 0.031$. Both tests reject the null hypothesis that the heart rate is the same under illumination by the different lasers for the standard significance level of 0.05. The difference in heart rate when the illumination source is changed is highlighted using normalized time series in Fig. 3(b) and in the box plot of all heart rates recorded using two-photon light sheet microscopy, which is shown in Fig. 3(c).

The increase in heart rate is due to the heating generated by laser illumination, since, upon switching to bright field illumination, the heart rate reverts back to its baseline value. Zebrafish heart rate has previously been established as a measure of laser-induced photo-injury in both single-photon [10,11] and two-photon light sheet microscopy [5], increasing (decreasing) linearly with a rise (fall) of temperature [14].

4. Conclusions and discussions

Multi-photon light sheet microscopy with an ultra-short pulse laser generating a propagation-invariant Bessel beam allowed us a large FOV while maintaining high axial resolution when compared to an equivalent Gaussian beam. Our study confirms that the laser repetition rate of the pulsed laser, and the consequent change in average power, is critical to minimise undesired thermal effects in light sheet imaging which may occur with high repetition rate, high average power sources. The zebrafish heart rate analysis clearly indicates that, even if in a low photobleaching regime, the ubiquitous use of a high repetition rate laser (e.g. 80MHz) for two-photon light sheet microscopy is far from ideal. The thermal effects produced by the laser illumination induces a clear deleterious increase of the zebrafish heart rate. By reverting to a 1MHz repetition rate laser with lower average power, we see a dramatically lower impact and a more representative recording of the true heart rate of an immobilised zebrafish.

Our conclusions implicitly assume that the exposure time is fixed to the one which optimizes recovery of the desired dynamical processes in the sample. In this case, for a given desired signal level we have shown that a lower repetition rate, lower average power laser is optimal for minimizing sample heating. We emphasize that to secure this advantage we should remain below any nonlinear photodamage or photobleaching threshold. In this regard Resan, *et al.*, [15] defined a figure of merit for nonlinear (multiphoton) imaging which, for two photon microscopy, is approximately the product of the peak power and average power. Here, a lower value of the figure of merit indicates reduced photodamage. For our data this shows a figure of merit for the low repetition rate source is approximately half that of the high repetition rate source (Table 1). This confirms the advantage of our approach to multiphoton imaging (see figure 4 in [15].)

Table 1 shows the exact laser parameters used in this study: notably, the heating generated by the lasers could be certainly decreased further, for example by correcting the pulse width of both Opera-F and Chameleon down to a 50fs range. To maintain the same fluorescence signal, the average power for each laser would be reduced and consequently also the effects on the zebrafish heart rate. However, the difference would be still significant as the pulse width of the Opera-F laser in our study was about 1.4 times larger than the Chameleon laser (Table 1). Alternative routes to minimise heating could involve reducing the FOV at the cost of compromising the imaging: this might be through the use of Gaussian beams, the use of a higher numerical aperture

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for the detection objective (the setup used a 20X 0.5NA Olympus objective) or a Bessel beam with a shorter DOF (e.g. changing beam size or axicon angle). Overall, our experiments confirm that in two-photon LSM laser repetition rate plays an important role. There is a trade-off between laser repetition rate, average power, thermal damage, photobleaching and photodamage that deserve due consideration in experimental design. Imaging in the linear (single photon) regime might also benefit from these considerations, though it is noted this would involve shorter penetration distances and the choice of laser power may result in deleterious heating and an increase of the recorded heart rate [10,11].

Increased thermal heating of a sample may also effect other physiological aspects of zebrafish. This might include oxygen consumption, gastrointestinal function and kidney development [16]. This would be worthy of investigation in future studies. Heating due to the use of high repetition rates has been seen in other biological systems: for example Gautam *et al.* show that using temporal gating to reduce the effective repetition rate (and thus average power) can maintain the viability of living neurons in brain tissue [17].

It is clear that laser repetition rate is a parameter that merits careful consideration for multi-photon light sheet microscopy experiments.

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Disclosures

The authors declare no conflicts of interest.

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