SHORT COMMUNICATION

Continuous wave excitation two-photon fluorescence microscopy exemplified with the 647-nm ArKr laser line

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Summary

We report on efficient two-photon fluorescence imaging in beam scanning microscopy by exciting UV dyes at the 647-nm line of a continuous wave ArKr mixed gas laser. For a numerical aperture of 1.4 (oil), we used an illumination power of up to 210 mW at the sample. High-resolution images were obtained for DAPI-labelled cell nuclei within 4–60 s. Our method is a simple two-photon alternative to UV confocal imaging with the potential of becoming a very useful feature of laser scanning microscopy.

Introduction

When first demonstrated (Denk et al., 1990), two-photon excitation microscopy (2PM) attracted great interest because of a series of advantages inherent in the excitation of fluorophores by simultaneous absorption with two (infra-) red photons. The benefits of 2PM include reduced photobleaching, deeper penetration into the sample and inherent three-dimensional imaging. Another tempting feature is the option to image UV dyes such as DAPI and Hoechst without being forced to use expensive UV microscopes.

While two-photon microscopy impressively succeeded in adding new dimensions to (neuro-) biological imaging of thick specimens and tissues (Denk & Svoboda, 1997), it has not yet really challenged the UV confocal microscope as a method for the 3D imaging of UV fluorophores. The reason is probably that practical two-photon excitation microscopy is thought to be possible only in conjunction with mode-locked lasers providing trains of subpicosecond pulses at a high repetition rate (Denk et al., 1995). The standard light source for 2PM is the mode-locked titanium–sapphire laser, with the all-solid-state Cr:LiSAF or Nd:YLF laser being used less frequently. The drawback of all these systems is that they are technically more complex and expensive than UV equipment, so they are not regularly an alternative for confocal microscopists.

Two-photon excitation microscopy with subpicosecond pulsed excitation can be motivated by the time-averaged two-photon molecular absorption rate:

\[ \sigma_2 = \frac{0.56}{\frac{1}{\tau} \left(\frac{\hbar \omega}{E}\right)^2 \sigma_1} = \frac{\sigma_0^2}{(\hbar \omega)^2 F^2 \sigma_2} \]

where \( \phi_2 \) is the time-averaged power, \( \tau \) represents the pulse duration, \( f \) the repetition rate of the pulses, \( \hbar \omega \) the photon energy, \( F \) denotes the focal area of the lens and \( \sigma_2 \) the two-photon cross-section. The factor of 0.56 holds for hyperbolic-secant shaped pulses. For mode-locked titanium–sapphire lasers (\( \tau \approx 250 \) fs at the sample, \( f \approx 80 \) MHz) we obtain \( \xi = 0.56/(\tau f) = 2.8 \times 10^4 \), while for a continuous wave (CW) lasers, \( \xi = 1 \). If the average power \( P_{\text{ave}} \) is kept constant the two-photon absorption rate \( \phi_2 \) increases by \( \xi \) so that pulsing appears necessary.

However, while the efficiency of pulsed two-photon excitation varies only inversely with the pulse width \( \tau \), it varies quadratically with the average power \( P_{\text{ave}} \). In principle, the absence of femtosecond pulsing can thus be compensated for by increasing the power by \( \sqrt{[0.56/(\tau f)]} = 167 \) so that \( P_{\text{ave}} = P_{\text{cw}} = 167 \) mW of CW light is as efficient as \( P_{\text{ave}} = 1 \) mW of the titanium–sapphire pulse train (Hänninen et al., 1994). The increase in average power in CW two-photon excitation microscopy (CW2PM) over its femtosecond pulsed counterpart (Fig. 1) is
accompanied by a reduction of the peak intensity at the sample by $\sqrt{0.56/(t_f)} < 167$.

The first realization of CW two-photon imaging goes back to Hänninen and Hell, who have succeeded in demonstrating CW2PM (Hänninen et al., 1994, 1995). Although independently confirmed and extended (König et al., 1995), these results had only a modest impact. One of the reasons was perhaps the low CW power used in these experiments, $P_{cw} < 3–10$ mW which could provide only a faint signal. Moreover, the experiments were carried out with a slow stage-scanning microscope unsuitable for investigating fast CW2PM. The recent acquisition of a standard beam scanning confocal microscope enabled us to investigate CW2PM under customary scanning conditions. Here, we provide evidence for efficient two-photon imaging with CW lasers using a microscope fitted with a standard beam scanning system.

Materials and methods

Our investigations were carried out using a standard beam-scanning microscope (TCS NT, Leica Lasertechnik, Heidelberg, Germany). In this system, the specimen slide is placed on a galvanometric $z$-scanning stage capable of 40 nm positioning accuracy; scanning in the $x$ and $y$ directions is performed by a galvanometric mirror (Fig. 2). A water-cooled argon–krypton laser (Coherent Innova 70C Spectrum) operating on the 647-nm wavelength (single mode, TEM$_{00}$) provided the illumination. First, the beam passed through a Schott RG610 long-pass filter, which effectively blocked any UV radiation emitted from the laser aperture. The excitation beam was coupled to the microscope scanning unit by a high-reflectivity mirror. We did not use an excitation pinhole because the large magnification factor of the scanner optics allowed us to expand the beam and overfill the aperture. In fact we compressed the beam waist by a factor of three (Fig. 2) to obtain a beam waist of 8–10 mm at the plane of the entrance pupil of the objective lens.

We used both water- and oil-immersion lenses of high numerical aperture (100× 1.4 oil, Leica PL Apo) whose entrance pupil (5.2 mm for the oil lens) was slightly overfilled. The fluorescence signal was collected by the same objective lens and then reflected by a short-pass dichroic mirror (edge at 580 nm) into a standard blue-sensitive photomultiplier operating in the analog mode.

We used nondescanned detection, that is the fluorescence light did not pass through the scanner optics. Rather we imaged the exit pupil of the objective lens onto the large area of the photomultiplier with a lens. By using nondescanned detection we excluded almost any potential confocalization through beam vignetting in the intermediate optics of the scanner. The scattered 647-nm light was removed by a filter block (F2 in Fig. 2). The filter block consisted of a pair of dichroic short-pass filters, each suppressing the 647-nm light by four orders of magnitude (KP600, Jenoptik, Jena, Germany). The dichroics were accompanied by two Schott BG39 filters of 2 mm thickness, each suppressing the laser light by 96.4%. The filters were arranged in the following order from the detector: KP/BG39/KP/BG39. The operation of the microscope and the data acquisition were carried out using the software that accompanies the microscope. Data from two-photon absorption cross-sections at 647 nm are not available, but measurements of $\sigma_2$ in the 680–720 nm range (Xu et al., 1996) leave room to envisage significant cross-sections for DAPI and Hoechst at 647 nm.

Fig. 1. Sketch of the different illumination conditions in pulsed and CW two-photon microscopy (not to scale).

Fig. 2. Set-up of the beam scanning CW two-photon microscope: laser, ArKr ion; filter F1, Schott RG610; filter F2, combination of dichroics and Schott BG39.
Results

To evaluate the 3D-imaging capability of our system, we first investigated CW2PM of beads. Among others, we used 9·7-μm latex beads (Molecular Probes, Leiden, the Netherlands) featuring maximum single-photon absorption at 365 nm and maximum fluorescence emission at 415 nm. The beads were mounted in glycerol and exposed to 180 mW of 647-nm CW laser light which was continuously scanned along a line in the x-direction for 10 min using the 100× oil lens. Figure 3 demonstrates the localization of bleaching observed using CW2PM at 647 nm. The images, taken using 60 mW illumination power, show that bleaching is confined to the focal region both along, and perpendicular to, the optical axis. The axial confinement of bleaching is evidence for its nonlinear nature.

We have measured the axial resolution of the microscope by recording the axial edge of a coumarin 138 solution. The dye was dissolved in immersion oil (Merck, Darmstadt, Germany). The axial resolution (the 20–80% resolution measurement) was found to be ≈0·8 μm.

As the next step, we measured the relationship between the illumination power and the intensity of the fluorescence emission for DAPI and coumarin 138. The DAPI was bound to the DNA of a Drosophila salivary gland polytene chromosome mounted in Moviol; the coumarin was dissolved in immersion oil (Merck). Illumination intensity was varied by the insertion of neutral density filters into the beam path. Illumination power was measured indirectly by coupling a fraction of the beam into a detector using a beam splitter. Eight scans of 128×128 pixels were taken of the same x–y plane and the intensity of the emitted fluorescence light was calculated as the average pixel value from these eight frames. The linearity of the relationship between pixel value and detected light intensity was also tested. For coumarin 138, we found a power relationship of 1·93, and for DAPI, the relationship was 1·79, as shown in Fig. 4.

Figure 5 shows CW two-photon xy images of a Drosophila salivary gland polytene chromosome stained with DAPI and mounted in Moviol. The image size is 59·2×59·2 μm² (512×512 pixels) and the captions show the relative...
nominal penetration depth into the sample. The power at the sample was $P_{cw} \approx 200 \text{ mW}$ and the total acquisition time per image was 53 s (including the scan dead time), i.e. 32 ‘medium’ speed scans of the TCS NT, corresponding to a total pixel dwell time of 32 μs. The series of $xy$ images exhibit the 3D-imaging capabilities of the 647-nm CW two-photon microscope which can be inferred from the various planes with various features changing with penetration depths.

Fig. 5. CW two-photon fluorescence images of $xy$ sections of a *Drosophila* salivary gland polytene chromosome at different penetration depths.
Figure 6 displays a more detailed view of the polytene chromosome in four different $xy$ images taken at 1 $\mu$m axial distance. The change in axial depth nicely reveals the spiral structure. The power at the sample was $P_{cw} = 210$ mW and the total scan time per frame (256 $\times$ 256 pixels) was 32 s.

Figure 7 shows a CW-two-photon 3D-data stack after volume rendering taken of DAPI-stained mouse fibroblast DNA fixed in a mitotic stage. The imaging conditions were $P_{cw} = 210$ mW and the acquisition time per frame was 32 s. The inset in Fig. 7 shows one of the $xy$ frames.

**Discussion and conclusion**

Figures 4–7 demonstrate the practicability of two-photon microscopy of the nuclear stain DAPI with lasers emitting a couple of hundred mW of power at 647 nm. The nonlinear nature of excitation is demonstrated both through the confinement of photobleaching of the fluorophore in the 9·7- $\mu$m latex bead and in the almost quadratic slopes of the dependence of the fluorescence flux of coumarin and DNA-bound DAPI on the excitation power. The slight deviation from the quadratic power law, of 1·93, measured for coumarin 138 in oil, and about 1·8 for DAPI, remains to be investigated, but the sectioning capability of the microscope is present. The measurement of the axial resolution of 0·8 $\mu$m proves the capability of producing high-resolution two-photon images in 3D. We think that the resolution can be improved by further increasing the beam waist at the entrance pupil of the objective lens. In our experiments, we restricted the beam diameter to a maximum of 8–10 mm in order to use the light economically. As a result, the effective numerical aperture was expected to be slightly lower than specified by the lens. In fact, the maximum available power at the focus of $P_{cw} = 210$ mW corresponded to about 320 mW of light shining on the rear side of the objective lens. It is interesting to note that we did not observe damage to the fixed specimens so that we are confident that the average power, if available, can be raised further still, to values higher than 210 mW at the sample.

The proposal of CW two-photon microscopy was based on the following reasoning. If the limits of the fluorescence yield were determined by nonlinear effects, or by saturation through ground state depletion, then the use of pulses, or of pulses below a certain duration $\tau$, would necessarily not be advantageous. Conversely, increasing the average power up to the 100–500 mW required for CW2PM could result in limitation by linear effects, such as optical trapping and heating through linear absorption (for example, of water). While heating of water can be excluded for most applications (Scho¨nle & Hell, 1997), trapping will probably limit the maximum usable power. This phenomenon, whereby an object is trapped by optical forces in the focal region of a high-aperture objective lens, is used as a tool in several fields, especially for the micromanipulation of biological specimens (Svoboda & Block, 1994). In the initial CW2PM experiments carried out with the slow scan stage-scanning
microscope, trapping had a noticeable limiting effect (Hänninen et al., 1995). In this study, we have overcome this problem by employing beam scanning which operates at about 1000-fold higher scan speeds than the stage scanning experiments. Trapping depends strongly on the object size and shape and the viscosity of the surrounding medium. We observed focal trapping of 1-μm-diameter fluorescent latex beads in both water and glycerol. The effect, however, was significantly reduced when we lowered the laser power or increased the scanning speed. We did not observe any optical trapping of biological specimens in either water- or glycerol-mounted samples. This is almost certainly due to the high scan speed, well above the mechanical resonance of these systems. We successfully extended our studies to the CW2PM of live cells with Hoechst-labelled nuclei, about which we will report elsewhere.

Our water-cooled ArKr mixed gas laser is, of course, less convenient to maintain than its low-power air-cooled counterpart with which standard commercial microscopes are usually equipped. (We acquired the water-cooled version also in order to have 12 visible laser lines for single-photon excitation.) Air-cooled ArKr lasers provide much less average power so that the two-photon excitation rates reported in this paper cannot be achieved with the standard ArKr lasers in commercial systems. However, power levels of a couple of hundred mW of continuous power are becoming available through CW high-power diode lasers emitting in the red and near-infrared regime. These lasers also offer the advantage of simple fibre coupling and launching into a microscope. One could imagine (simultaneously) using laser diodes at 680 nm (e.g. AlGaInP), at 810 nm (e.g. GaAlAs) and, say, 980 nm (e.g. InGaAs). In quite a few high-aperture applications, a good compromise between cost-efficiency and signal is anticipated.

In CW2PM, compared to subpicosecond pulsed excitation, the use of an N times higher CW power is accompanied by an N times lower maximum (peak) power. While the limitations of subpicosecond pulsed two-photon imaging are probably due to the nonlinear processes involved with the high peak power, the limitations of CW two-photon

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**Fig. 7.** CW-two-photon excited 3D-data stack after volume rendering taken of DAPI-stained mouse fibroblast DNA undergoing mitosis taken with 210 mW. The acquisition time per frame was 32 s; the inset shows one of the xy frames.
microscopy will most likely stem from trapping and from the limited availability of excitation light. Potential natural fluorochromes with single-photon absorption in the infrared range could also impede the use of CW excitation, but they might not be very common. As significant two-photon absorption is possible, limiting by nonlinear effects might also occur, as is also the case with pulsed excitation. Our results have a further implication: the successful use of 210 mW at the focus of a high-aperture lens (NA = 1.4 oil) suggests that nonlinear effects may be serious candidates for the limitations observed with pulsed two-photon microscopy at similar and lower average power. In other words, the average power used is not conclusively the limiting factor.

To summarize, we have found that efficient high-aperture two-photon microscopy of UV dyes can be carried out with CW excitation at 647 nm. Our results open up the prospect of two-photon imaging with CW laser diodes, whose potential and limitations at other wavelengths will be highly interesting to explore.

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References