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Three-dimensional image formation under single-photon ultra-short pulsed illumination

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Abstract

The major thrust of modern day fluorescence laser-scanning microscopy have been towards achieving better and better depth resolution embodied by the invention and subsequent development of confocal and multi-photon microscopic techniques. However, each method bears its own limitations: in having sufficient background fluorescence and photo-damage resulting from out-of-focus illumination for the former, while low multi-photon absorption cross-sections of common fluorophores for the latter. Here we show how the intelligent choice of single-photon ultrashort pulsed illumination can circumvent all these shortcomings by exemplifying the tiny spatial stretch of an ultrashort pulse. Besides achieving a novel way of optical sectioning, this new method offers improved signal-to-noise ratio as well as reduced photo-damage which are crucial for live cell imaging under prolonged exposure to light.

Keywords

Fluorescence microscopy; optical sectioning; confocal detection; multi-photon excitation; pulsed illumination; ultra-short pulses; second-harmonic generation; threshold detection

1. INTRODUCTION

Scanning microscopy has revolutionized the study of tiny structures visually in real time. New scanning microscopic techniques have been invented over time with vast range of applications. Still, the highly resolving electron microscopic techniques, on one hand need, sample preparation which renders them obsolete in live cell imaging. While, on the other hand, the highly deep tissue penetrating long wave-length radio-frequency based magnetic resonance imaging is impaired by the fundamental diffraction limited resolution. Microscopy at optical wavelengths, thus, seems to combine both ends together in having better resolution as well as being bio-friendly. Among different microscopic techniques fluorescence microscopy has evolved as the most widely applicable tool for live cell imaging by probing fluorescently tagged molecules as well as intrinsic fluorophores inside a live cell. Confocal [1] and two-photon [2] microscopy are two techniques which impart better depth resolution in an 'optically thick' (thickness >20 microns) specimen. However, the presence of sufficient background fluorescence has been the major drawback in confocal

detection while the very low two-photon absorption cross-section has restricted the wider use of the latter technique.

Here we describe our recent work [3] on fluorescence laser-scanning (galvo-mirror scanning) microscopy with femto-second single-photon pulsed excitation that indeed leads to a consortium between confocal and multi-photon microscopy.

2. METHODOLOGIES

In our experiment, the laser system was a mode-locked Ti:saph laser (Mira900-F pumped by Verdi5, Coherent) producing femtosecond laser pulse trains at 76 MHz repetition rate having tunability in the range of 720-980 nm. We used ~150 fs pulsed excitation centered on 780 nm and frequency-doubled the light by a second-harmonic generation (SHG) crystal (type-1 beta-barium borate or BBO, Castech) to get a pulsed output at 400 nm. The ultra-short pulsed light beam (the fundamental or the second-harmonic) was sent to a multi-photon-ready confocal microscope system (FV300 scan-head coupled with IX71 inverted microscope, Olympus); the SHG beam was passed through an IR cut-off filter to ensure that no fundamental light is present). The 488 nm CW beam from an argon-ion laser (IMA 10X, MellesGriot) was used for confocal imaging. For imaging purposes, all slides were purchased from Molecular Probes and all images were taken using an oil-immersion objective (UPlanApoN 60X 1.42NA, Olympus) with 512×512 pixel size. For axial resolution measurements 10^{-4} M Rhodamine-6G (R6G) solution (in methanol) was used. All chemicals were purchased from Sigma-Aldrich.

3. RESULTS AND DISCUSSIONS

Figure 1 shows the relative point spread curves (obtained as Gaussian fits to the derivative of sigmoidal curve resulted from axial scanning [4]) for confocal microscopy; the black curve represents resolution keeping the confocal pin-hole (or aperture) intact and the red curve results when the pin-hole is removed. The blue curve is for two-photon excitation. Two points can be noted from the graph; firstly, the axial resolution is better in two-photon excitation and secondly, removing the pin-hole affects the axial resolution in one-photon CW illumination. Also, at far ($4\ \mu\text{m}$) from focus the intensity never goes to zero for confocal detection as there exists a lot of residual background fluorescence which is altogether absent in two-photon excitation. However, this is not the case for the one-photon pulsed illumination. As shown in figure 2, although the resolution is poorer than confocal counterpart, removing the confocal pin-hole almost leaves the depth profiling unchanged which means the technique has intrinsic three-dimensional (3D) spatial resolution. Also at far from focus, there is almost negligible background contribution even when the aperture was open. The use of tight focusing objectives as well as short wavelength illumination ensures lateral resolution; therefore the 3D resolution is imparted by the depth resolution of the technique. This is achieved due to the following reasons; as the ~100 femto-second pulse crosses the double-cone region across the focus it creates an image at the back focal plane of objective at every instant. However the sharpest of these images is the one when the pulse is just positioned at the focal plane [5]. At this instant, since the pulse is only stretched over few microns in space [6], the sharpest image has almost no out-of-focus contribution. If we

somehow collect only this sharpest image (which is back-ground free) we should, in principle, get 3D spatial resolution. This can be easily done by using the photo-multiplier gains in such a way that it collects only the signal above a certain threshold value corresponding to the sharpest signal [see figure 5 in reference 3]. However, it must be kept in mind that this will result only when sufficient amount of photons reach the detector which is normally true for the fluorescently labeled sample used in our experiment. We wish to call this technique as *detector thresholding*.

Signal enhancement under pulsed illumination has been thoroughly studied by our group [7]. The results revealed that one-photon pulsed illumination is better compared to continuous illumination as long as the pulse does not lead to any saturation effect which is common with sub pico-second pulses. To get rid of this, we probed at wavelength regions where the sample has minimum one-photon absorption. However, this does not affect the signal-to-noise ratio as the low absorption cross-section is circumvented by the huge photon density within an ultra-short pulse. Such illumination also removes solvent induced heating effects having direct applications in imaging which has been discussed in detail elsewhere [8, 9].

The advantages of single-photon pulsed illumination (SPPI) are far reaching. The second harmonic signal spanning the broad spectral window from 340 nm to 540 nm (for our laser system, this range is 360-490 nm) achievable from Ti:saph lasers using high pump power (~20 W green) covers the one-photon absorption region of most commonly used fluorophores. The corresponding third harmonic generation (THG) yields deep ultra-violet (UV) excitation which is necessary for many materials science applications but renders adverse conditions for live specimens under observation. Moreover, this entire spectral range is continuously tunable owing to the broad fluorescence curve of Ti^{3+} ions; therefore we can use only a solitary laser source for multiple fluorophores instead of several single line laser sources for each fluorophore. Additionally, this allows us to switch between multi-photon and single-photon microscopy at our will with the same multi-photon ready confocal microscope system coupled with a near-IR pulsed laser just by changing the SHG (or THG) crystal and IR filter. However, one disadvantage is that the visible window has lesser tissue-penetration capabilities as compared to the IR light. Otherwise, this illumination scheme combines all the best features of both confocal and multi-photon microscopy.

Figure 3 shows the comparative illumination schemes of the three techniques of fluorescence LSM. The SPPI technique, like the two-photon excitation scheme, is free from back-ground illumination (as evident from figure 2) while the confocal detection needs a pin-hole (marked by the central ellipse in figure 3) for the in focus fluorescence detection. Although the present method lacks better axial resolution as compared with the other two methods, pulse compression at the sample (as well as using few-cycle optical pulses) is promised to impart better optical sectioning capabilities. These issues are presently being pursued with the help of concave grating based laser pulse shaper in our laboratory.

4. CONCLUSIONS

We show how an intelligent approach of pulsed illumination leads to three-dimensional spatial resolution with enhanced signal-to-noise ratio as well as reduced photo-thermal

effects. The work shows, as a future direction, even shorter pulses should lead to better depth resolution; for example, atto-second pulses can lead to three orders of magnitude more resolution and can be used for nano-scale measurements. However shorter pulses come with the expense of gigantic peak powers restricting their applications to material science only and special care must be adapted for live cell imaging.

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Fig. 1.
Point-spread curves for axial resolution measurements in confocal and two-photon microscopy.

Fig. 2.
Point-spread curves for axial resolution measurements during single-photon pulsed illumination.

Fig. 3. Comparisons among three different laser-scanning fluorescence microscopic techniques (schematically); the fluorescence is shown in the darker shade (green color).