

Improved depth resolution in video-rate line-scanning multiphoton microscopy using temporal focusing

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By introducing spatiotemporal pulse shaping techniques to multiphoton microscopy it is possible to obtain video-rate images with depth resolution similar to point-by-point scanning multiphoton microscopy while mechanically scanning in only one dimension. This is achieved by temporal focusing of the illumination pulse: The pulsed excitation field is compressed as it propagates through the sample, reaching its shortest duration (and highest peak intensity) at the focal plane before stretching again beyond it. This method is applied to produce, in a simple and scalable setup, video-rate two-photon excitation fluorescence images of *Drosophila* egg chambers with nearly 100,000 effective pixels and 1.5 μm depth resolution. © 2005 Optical Society of America

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The field of multiphoton microscopy has seen great advances in recent years, since the introduction of the two-photon excitation fluorescence microscope.¹ The main advantage of this microscope is its inherent optical sectioning capability, as out-of-focus signals are practically eliminated by their nonlinear dependence on the illumination intensity. A major drawback of multiphoton microscopy is, however, the relatively long requisite image-acquisition time that is due to mechanical scanning and the serial acquisition of image points.

Numerous methods have been developed to increase the image-acquisition rate in multiphoton microscopes. Most involve multipoint illumination and scanning in a single spatial axis. Common examples are single-axis scanning and the use of line illumination,^{2,3} rotation of a lenslet array,^{4,5} and multipoint illumination with beam-splitter arrays.⁶ Line illumination is both easily scalable and the simplest to implement, but it suffers from reduced depth resolution, as the beam is focused only along one spatial axis. The main drawback of lenslet arrays is the increased complexity of the microscope setup. Beam-splitter-based systems are not easily scalable and suffer from point-to-point intensity variations. Here we describe a method that combines the simplicity of line scanning with the enhanced depth resolution of point scanning by focusing the excitation pulse not only along one spatial dimension but also temporally.

Recently it was shown⁷ that depth-resolved multiphoton microscopy can be performed in a scanning-less setup. This is done by temporal focusing of the excitation pulse rather than by spatial focusing. Briefly, the illumination beam excites part of (or all) the frame of interest in the specimen, an area that can be greater by orders of magnitude than the diffraction-limited spot of the objective lens. The depth resolution is achieved by control of the temporal profile of the pulse, which is compressed as it propagates through the sample, reaching its shortest duration at the focal plane and stretching again as it propagates beyond it. The depth-resolved multipho-

ton signal can now be collected from the entire illuminated frame by use of an imaging setup. In the research reported in Ref. 7 the temporal focusing was achieved by use of a diffraction grating that is imaged onto the object plane through a high-magnification telescope, one of whose lenses is a high-N.A. objective. The grating has the advantage that it can be designed to maximize the diffraction toward the microscope's optical axis when it is illuminated from an angle. This geometry is reminiscent of

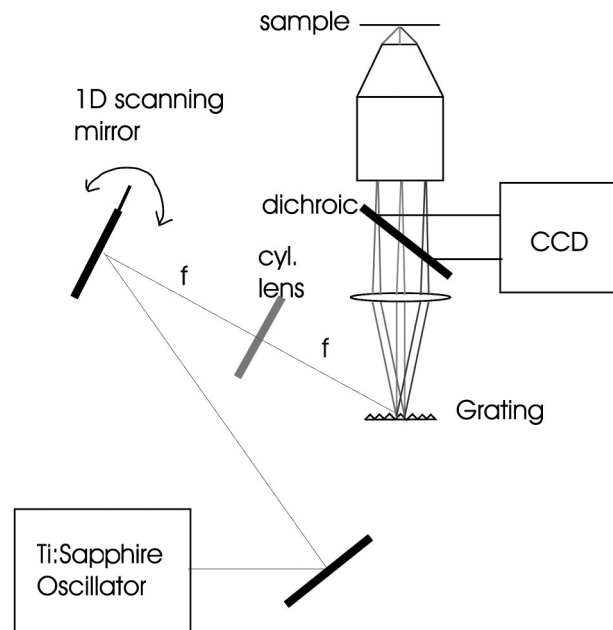


Fig. 1. Experimental setup: After passing through a grating compressor to compensate for dispersion and through a cylindrical (cyl.) $2\times$ magnifying telescope, the input beam is scanned and focused in one dimension (1D, not shown) on a grating, aligned perpendicular to the optic axis of the microscope. The grating is imaged onto the sample through a high-magnification telescope that comprises an achromatic lens and the microscope objective. Fluorescence is epidetected and imaged onto a CCD by a dichroic mirror.

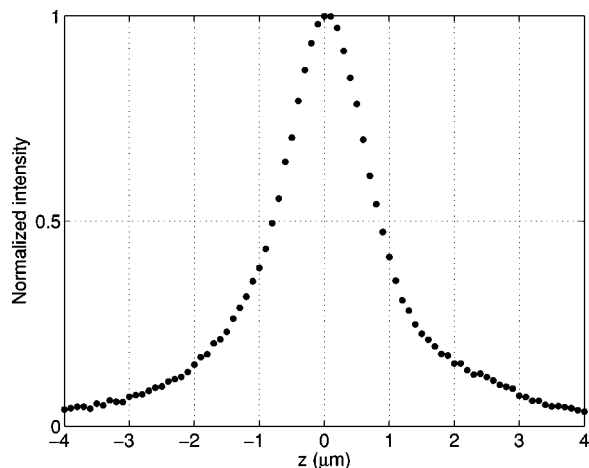


Fig. 2. Depth resolution of the TPEF microscope. The total fluorescence intensity is measured from a $0.8 \mu\text{m}$ thick spin-coated fluorescent layer as a function of its distance from the focal plane of the objective. Fluorescence from a line with dimensions of $80 \mu\text{m} \times 0.5 \mu\text{m}$ was collected and measured with a photomultiplier tube.

line-scanning microscopy, except that the full frame is scanned in a few picoseconds by the traveling pulse. Indeed, the depth resolution achieved with this method is similar to that of line-scanning illumination and is lower than that of the standard two-dimensional scanning method.

In this Letter we show that by illuminating the grating with a thin line (perpendicular to the grating lines), instead of an area, we can reach a depth resolution similar to that of nonconfocal point scanning two-photon excitation fluorescence (TPEF) microscopy. To get the full field, we mechanically scan the line across the grating, but, as mechanical scanning is reduced to one axis, it can be performed at much higher rates. For optimal depth resolution, the line illuminating the grating has to be tailored in such a way that as it travels on the grating it will be imaged through the telescope to produce a diffraction-limited spot traveling in the objective focal plane. This setup can easily achieve video rate, yet the depth resolution is comparable with that of standard nonlinear microscopes.

Figure 1 shows the experimental setup. It consists of a Ti:sapphire laser oscillator delivering 10 fs pulses at a repetition rate of 75 MHz. The pulses are then negatively chirped by a grating compressor⁸ to compensate for the dispersion of the lenses in the system and are horizontally expanded by a $2\times$ cylindrical telescope. They are then scanned by a galvanometric scanner and focused by a 20 cm cylindrical lens to produce a line of approximate dimensions of $10 \text{ mm} \times 40 \mu\text{m}$ on a 300 line/mm grating aligned perpendicular to the microscope's optical axis. The focused line is perpendicular to the grating grooves. The telescope consists of a 20 cm achromatic lens and a $100\times$, N.A.=1.3 Fluar objective (Zeiss), corresponding to a magnification of $\sim 125\times$. This setup produced fields of view of $\sim 80 \mu\text{m}$ in the unscanned axis and $\sim 120 \mu\text{m}$ in the scanned axis in the object plane. The total illumination energy per pulse was

$\sim 0.15 \text{ nJ}$, corresponding to an energy per pixel of the order of 2 pJ (of the order of a stringent assessment of the damage thresholds⁹). As an imaging platform we use a Zeiss Axiovert inverted microscope. Epifluorescence was imaged onto a cooled intensified CCD via a dichroic mirror and a filter that rejects the excitation light. For depth resolution measurements we used a photomultiplier tube and a lens to collect all the fluorescence from a single layer.

First we demonstrate the depth resolution of the method described by measuring the total fluorescence signal obtained from a $0.8 \mu\text{m}$ layer of a two-photon fluorescent dye (Coumarin 515) in a polymer matrix spin-coated onto a glass slide, as a function of its position relative to the objective focal plane. The result of such a scan is plotted in Fig. 2. The measured FWHM is $\sim 1.7 \mu\text{m}$, which, after deconvolution of the layer thickness, gives a FWHM of $1.5 \mu\text{m}$. This is close to the value of $\sim 1 \mu\text{m}$ obtained by Stelzer *et al.*¹⁰ and is better than the confocal line-scanning depth resolution ($\sim 3 \mu\text{m}$) reported by Brakenhoff *et al.*²

In the imaging experiments we observed *Drosophila* (fruit fly) egg chambers at an early develop-

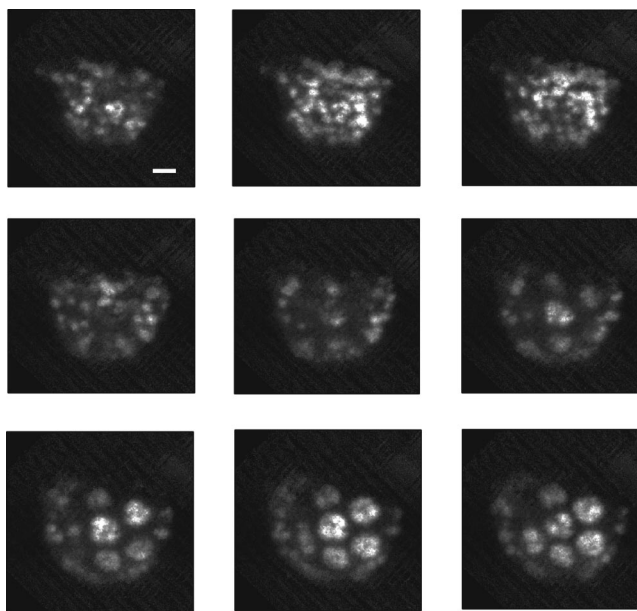


Fig. 3. Depth-resolved images of a *Drosophila* egg chamber stained with DAPI. Optical sections of a *Drosophila* egg chamber containing 15 nurse cells and a single oocyte and wrapped by a layer of follicle cells are presented. The images go from the bottom of the egg chamber (top left image) to the center of the egg chamber (bottom right image). The approximate area of each image is $100 \mu\text{m} \times 80 \mu\text{m}$. The scale bar is $10 \mu\text{m}$. Images are slices separated by $2 \mu\text{m}$. The integration time for each image was $\sim 100 \text{ ms}$. The intensifier noise was subtracted from each image, which was corrected for spatial variations in the beam intensity, assuming a Gaussian beam profile. In the top row, follicle cells, whose nuclei are approximately $3 \mu\text{m}$ in diameter, are shown. The images in the bottom row show the nuclei of nurse cells, whose size is of the order of $10 \mu\text{m}$, as well as the enveloping follicle cells.

mental stage and stained with 4',6'-diamidino-2-phenylindole hydrochloride (DAPI), a fluorescent groove-binding probe for DNA whose absorption band is centered at 400 nm. Figure 3 shows nine optical cross-sections, $2\ \mu\text{m}$ apart, taken at 100 ms per frame. Each egg chamber comprises 15 relatively large nurse cells and a single oocyte, which are surrounded by a coating of small follicle cells. The lateral resolution of the images, determined by the N. A. of the objective, is of the order of $0.25\ \mu\text{m}$. The total area covered is $\sim 10^4\ \mu\text{m}^2$, corresponding to $\sim 10^5$ effective pixels.

The laser used in our experiment is not optimal for this application in terms of repetition rate, bandwidth, and power. A more detailed discussion is given in Ref. 7. Optimally one would prefer a source of smaller bandwidth ($\sim 30\ \text{nm}$) to better match the fluorophore absorption spectrum and to be less sensitive to chromatic aberrations and material dispersion. Furthermore, somewhat higher peak powers would permit the use of lower acquisition times and an easily scalable larger field of view. Such pulses are currently available in commercial systems. In particular, extended-cavity lasers, which deliver higher energy per pulse at the cost of a reduction of the repetition rate, are highly suitable for imaging large areas by this method.

In summary, we have shown that one can produce TPEF microscopy images at video rate and beyond, at a high depth resolution (similar to that achievable by point scanning), by employing ordinary line scanning in the lateral dimension and temporal focusing for enhancing depth resolution. The suggested method utilizes only simple, off-the-shelf components and does not require any modification to the microscope itself. More importantly, this method is not unique to TPEF microscopy but can be used with practically

any multiphoton process, such as second-harmonic generation,¹¹ third-harmonic generation,^{12,13} or coherent anti-Stokes Raman scattering.^{14,15}

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