Real Time 3D fluorescence microscopy based on multifocal multiphoton excitation

Ramesh Ahuja, TauTec LLC

Multiphoton excitation microscopy is becoming increasingly important as it offers several advantages over confocal laser (single photon excitation) scanning microscopy (CLSM) such as use of lesser damaging NIR wavelengths, reduced scattering, deeper imaging, no need for pinhole, higher light collection efficiency and excitation of UV dyes at NIR wavelengths; in addition, excitation and photobleaching are limited to the focal region.

Multiphoton microscopy shares one disadvantage with CLSM, though- low image rate. Images must be reconstructed pixel by pixel, and with an image rate of 1-2/s, the technique is not suitable for investigating fast biological processes. To speed up image acquisition, various techniques such as line scans, polygon mirrors, resonant scanners and microlens arrays have been used.[1] The problems with the microlens array design are: low light throughput, non-uniform intensity foci and lens aberrations. In our approach[2] a special beamsplitter, based on flat optical elements, divides an incoming laser beam into N beamlets that are coupled to the microscope and scanned simultaneously in the object plane, speeding up image acquisition by a factor of N; depending on the laser power, N can be as high as 256.

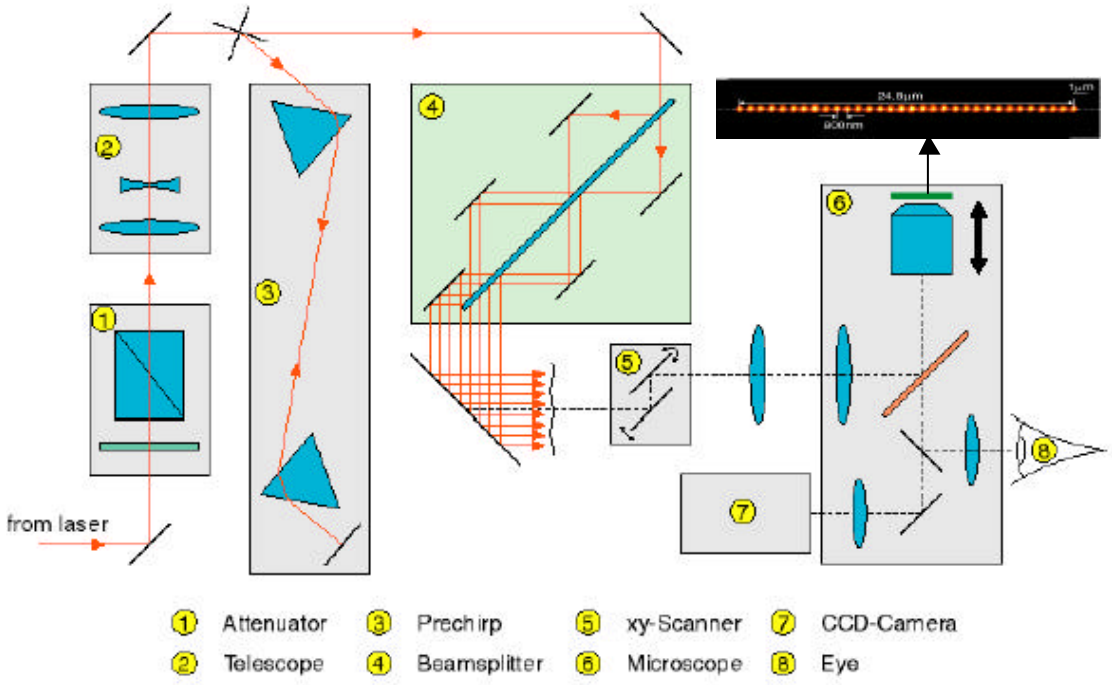

Fig. 1 optical setup of a multifocal multiphoton microscope based on simultaneous scanning of 64 beamlets. The inset (linear array) shows two-photon excited fluorescence of Rhodamine 6G dissolved in water.

The beamsplitter module (block 4 in fig. 1) consists of a set of wide-band reflecting (bold black lines) and semireflecting (bold blue line) mirrors that generate two sets of 32 beamlets which are combined into a linear array of 64 beamlets with alternating polarization states (s-p-s-p..). Several important features of the beamsplitter are: higher (>75%) light throughput, uniform intensity of beamlets, intrinsic time-multiplexing due to slightly different optical paths, absence of optical aberrations due to flat optical elements, and convenient control on the polarization, degree of parallelization (number) of beamlets, inter-foci distance and field of view. The outgoing beamlets are coupled into

the microscope via an XY scanner (block 5 in figure 1) and intermediate optics; a microscope objective focuses the beamlets into multiple foci on to the object plane.

The optical system as shown in fig. 1 yields a linear array of foci with an adjustable inter-foci separation in the 0.4...2µm range. The XY scanner allows the positioning of the foci-array anywhere within the field of view of the microscope objective. The scanner can be operated either in the resonant or non-resonant mode. Scanning in the XY plane generates a plane of two-photon excited fluorescence; in combination with the Z-drive coupled either to the microscope objective or stage, high resolution 3D fluorescence images can be obtained. Although the two-axis galvanometer scanner can achieve frame rates of up to 3500 Hz, which can accommodate real-time observation and acquisition of 3D stacks, the final frame rate of the imaging system is limited by the readout speed of the CCD camera and the intensity of fluorescence signal.

The beamsplitter can be configured to generate either a linear or area array of beamlets. The area array can be generated by coupling the output of the first stage beamsplitter via a periscope to the input of the second stage beamsplitter. The linear array, however, offers several advantages: in addition to the standard XY-scanning mode, it also allows the acquisition of XZ-planes (see figure 2) and convenient coupling to the input slit of an imaging spectrograph enabling high speed spectral imaging and sectioning.

The number of beamlets in the linear array can easily be switched from 64 to 32,16,8,4,2 or a single beam by moving the semi-reflecting mirror in and out of the optical path. This option is quite important when imaging deeper into the sample where due to scattering losses, higher laser intensity is required for fluorescence excitation.

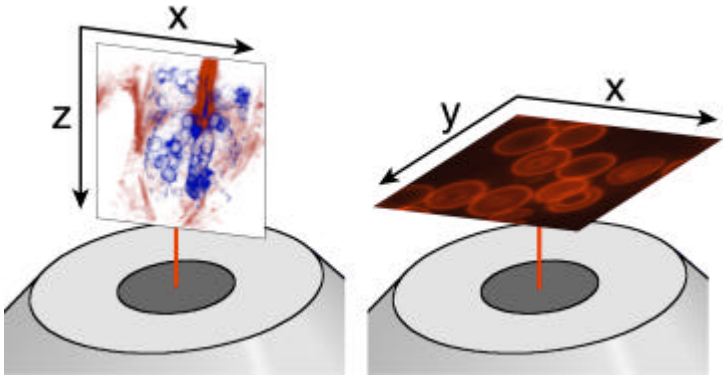

Figure 2: scan modes for fast imaging of x-y and x-z planes.

By changing the intermediate optics, the distance between neighboring foci can be reduced to the extent that individual foci start overlapping. As a result the sample is illuminated homogeneously along a shortened line enabling line-imaging without the need for scanning.

As a result of broad bandwidth of 100fs input pulse, the pulse undergoes dispersion during its passgae through the various optical elements on its way to the object plane resulting in pulse broadeing. The pusle width increases typically from 100fs to 600fs. The probability of two-photon absorption is inversely proportional to the pulse width. In order to optimize the two-photon excited fluorescence signal, it is desirable to introduce a prechirp optics as shown in block 3 in fig. 1.

Compared to the approach based on spinning microlens array[1], the design discussed here has higher optical throughput and image rate by a factor of 10 while providing equally intense foci with adjustable interfoci distances and minimal interfoci cross-talk. It also allows motorized control on the degree of parallelization (number of foci), polarization (foci polarization pattern: s-p-s-p...) and intensity (block 1 in fig. 1) of beamlets. As the

microlensses can not be fabricated aberration-free, the axial resolution is not diffraction limited.

A **m**ultifocal **m**ultiphoton **m**icroscope (MMM) is ideally suited for whole field 3D **F**luorescence **L**ifetime **I**maging **M**icroscopy (FLIM)[3]. By coupling a picosecond gated, high rep. rate image intensifier (PicoStar HR; 200ps gate width @ 110 MHz rep. rate) to the CCD camera in fig. 1, one can acquire a series of time-gated images at various delay values with respect to the laser pulse and generate FLIM image. Thus MMM by itself and especially in conjunction with multimodality (FLIM, FRET, FRAP, spectral and anisotropy) imaging, opens up new possibilities for real time 3D high resolution investigation of intracellular processes, membrane trafficking, Ca^{++} signaling, particle tracking, tissue screening and protein-protein interactions.

References

1. J. Bewersdorf, R. Pick, S. W. Hell: Multifocal Multiphoton Microscopy, Opt. Lett. **23**, 655-657, (1998).
2. T. Nielsen, M. Fricke, D. Hellweg, P. Andresen High efficiency beamsplitter for multifocal multiphoton microscopy, J. Microscopy. **201**, 368-376 (2001).
   US Patent no. 6219179
3. M. Straub, S. W. Hell: Fluorescence lifetime three-dimensional microscopy with picosecond precision using a multifocal multiphoton microscope, App. Phys. Lett., **73**, 1769-71, (1998)

About the author

Ramesh Ahuja, PhD is president of TauTec LLC, 9140 Guilford Road, Suite O, Columbia, MD 21046. Phone: (301) 725 7441; fax: (301) 725 2941; email: rahuja@tautec.com