Construction of a Conventional Two-Photon Excited Fluorescence Microscopy

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Abstracts: We construct a point scanning two-photon excited fluorescence (TPEF) Microscopy. We used it to image fluorescent beads and nucleus of neurons. Furthermore, we improve the field of view up to the size limited by the mirrors.

1. Introduction

In 1990, Denk first demonstrated TPEF microscopy. It has the axial sectioning function similar to a confocal microscopy while normal microscopy does not have the sectioning function.[1,2] Thus it offers us another way to get three dimensional imaging. Compared with confocal microscopy, TPEF microscopy has simple setup which does not require pin-hole detector and fine alignment for it. On the other hand, TPEF microscopy need longer wavelength for two-photon excitation so that the axial and lateral illumination resolution is almost half of the value for confocal microscopy.[2] The advantage is that due to the longer wavelength, the absorption and scattering of the incident light is low and thus it's better for deep imaging. Moreover, although the illumination resolution in axial direction is worse for TPEF microscopy, the far field axial background has the same shape which is $1/z^2$ as the confocal microscopy.[3] So they have the same deep imaging ability in terms of axial background level. All in all, since TPEF microscopy utilizes nonlinear optical effect which is weak, usually we use a pulse laser instead of CW laser to be the light source in order to enhance the nonlinear effect.

In our work, we utilized the Galvano scanning mirror to do two dimensional lateral-scanning. The maximum frequency of the Galvano mirror is around 500Hz and we operate the system to get one image from one to ten seconds. Quick imaging rate limits the highest spatial frequency we can achieve so that we need to tradeoff between imaging rate and image quality. And later, we improved the setup in order to prevent the limitation of field of view due to scanning. We utilize a 4-f system to relay the change of the wavefront on the Galvano mirror while we do not need to move the beam itself. Thus we can get the field of view up to the size limited by the mirrors.

2. Experimental setup

A mode-locked Ti:Sapphire laser (Venteon) with an average power of 180mW and a repetition rate of 150 MHz works as a light source. The bandwidth is around 400 nm and the central wavelength is 830nm. Spectrally phase modulated for dispersion compensation by the pulse shaper, the laser source has an average power more than 30mW. The laser beam is first aligned by two mirrors and two iris pair. Then the laser goes into a 2-D Galvano Mirror (T.E.M. Incorporated 6230HM50A). Incident laser will pass the dichroic mirror (DM) and focused by an objective lens (OBJ). Additional two irises are used for fine alignment while we do not need to do that for daily use. Two-photon fluorescence light is collected by the same OBJ and reflected by DM (high transmittance~400nm). If the fluorescence light has enough power, we can directly see the image on the CCD by a tube lens of 200mm focal length. However, for daily use, we use another flipping mirror to reflect the fluorescence light into PMT and rebuild the image by Labview program.

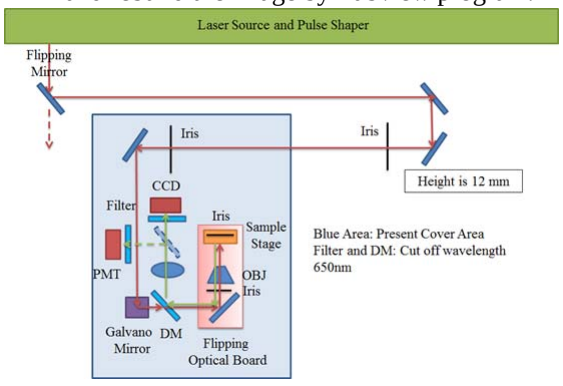


Figure 1 Experimental setup

In order to check the alignment is good enough for daily use, we used common incoherent light source to illuminate light from the focal region of OBJ. For incident light, if it is well aligned, when we put a white paper at the back aperture of OBJ as a test screen, the laser spot should locate at the center of the envelope of the incoherent illumination light. And definitely we will set the Galvano mirror at zero degree for this test. For the collecting system for TPEF signal, we can still utilize the envelope of the incoherent illumination light as the reference for the position of TPEF light. As long as the envelope of the incoherent illumination light is geometrically covered by the DM, filter and PMT, we think the system is well aligned. For the alignment of CCD camera, we only need to make sure the distance between CCD photocathode and the tube lens is one focal length by searching the image whose related object is far away from the tube lens.

3. Experiment Results

3.1 Steps for operation

Due to the cut-off wavelength of filter which is around 650nm to 680nm, we have to use the wavelength above 700nm for the incident light by blocking the unnecessary frequency components on the Fourier plane of the pulse shaper. Then, based upon previous experience, we first set the pulse shaper to roughly compensate the dispersion of the microscopy system. Then we hold the sample on the moving stage and search the image on CCD via the incoherent back-illuminated light. In this process, we can choose the area where the interesting object locates. For testing image of fluorescent beads whose diameter is 1μm, we can choose the area where half of the region is fluorescent beads because the big and obvious spatial change will easily be recognized from the TPEF image either on CCD or by PMT even if the signal is low or the axial position is not fine-aligned. Note that the region of fluorescent beads is the dark or black region since light is blocked from backside. We think the axial position of sample for image by incoherent light, laser scattering and TPEF is not at the same position but very close which is less than several hundred micrometers. Thus when we switch to the TPEF imaging mode, we just need to slightly change the axial position of sample by hand. As long as we find that the signal is suddenly increasing, we think we are in the focal region which is around several micrometers and around axial FWHM of TPEF intensity convolves with the size of fluorescent beads. For fine alignment, we can use pico-motor to do the changing of every 1 μm in axial direction.

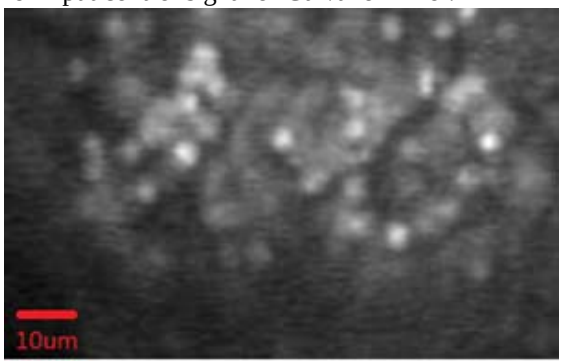
3.2 TPEF images and performance

As Figure 2 shows, we took the TPEF image from a real biological sample and also testing fluorescent beads. For testing fluorescent beads, it shows different lateral resolution by different OBJ with different N.A.. Once the N.A. is 0.95, we can clearly see the single fluorescent beads which are circles with diameter of 1μm. One interesting phenomena is that once we increase the imaging time as well as scanning period with the same rate, the output signal does not increase linearly. I think this is due to the dark state which decreases the TPEF intensity with excitation time.

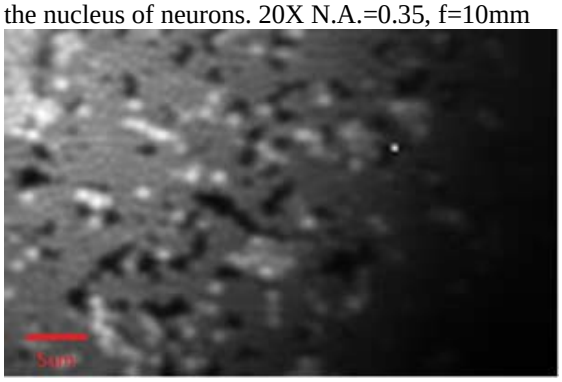
Our Galvano mirror has horizontal (X) and vertical (Y) scanning mirrors. Horizontal mirror is operated by a 20Hz triangular wave with a moving range of +-0.4 degree. Linear range is more than 80% of the total range. Thus we only use a moving range of +-0.32 degree as imaging range. Vertical mirror is operated by a 0.1Hz triangular wave with a moving range of +-0.2 degree. Linear range is more than 99.9% percentage of the total range. Note that if the data acquisition bandwidth is higher enough, we can increase the scanning speed five to ten times with the same parameters. The present scanning speed can offer us a highest imaging rate of 1 frame/sec but the image quality is limited. Moreover, we can increase the moving range with a loss of resolution due to data acquisition speed. On the other hand, the moving range for the setup in Figure 1 is limited since we have to move the excitation light at the back aperture of OBJ which means big moving angle will let us lose a lot of power and effective N.A..

Imaging speed is from 5sec to 10sec, which

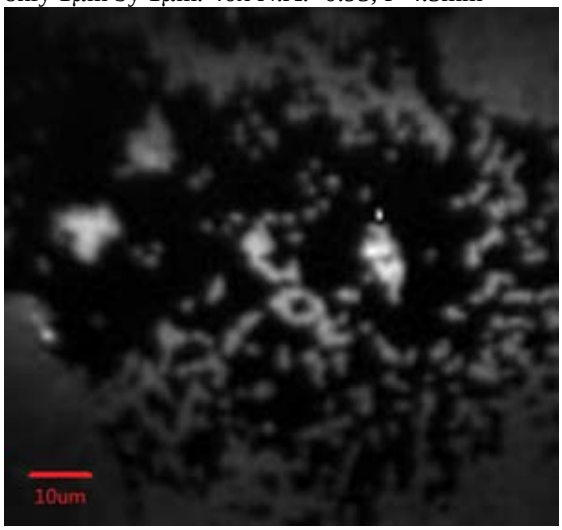
means the frame rate is 0.1 to 0.2 frame/sec. Note that the imaging speed now is limited by the data acquisition rate rather than laser power or scanning speed. Present DAQ board has a bandwidth of 250K Sample/sec. Three output channels including X position, Y position and image signal each has a data acquisition rate of 50K Sample/sec. The remaining bandwidth of 100K Sample/sec is saved for input control signal of Galvano mirror.



(a) TPEF image of Fluorescent beads (358/461) in the nucleus of neurons. 20X N.A.=0.35, f=10mm



(b) TPEF image of Fluorescent beads (430/465) only 1μm by 1μm. 40x N.A.=0.95, f=4.5mm



(c) TPEF image of Fluorescent beads (430/465) only 1μm by 1μm. 20x N.A.=0.35, f=10mm, angle of incident light optimized

Figure 2 TPEF images

Imaging size is determined by the focal length of the objective lens. For example, a 20x Nikon objective lens has a 10mm focal length and the whole imaging size is 112μm by 70μm. This image is digitized to 160pixle by 100pixel. Note that the vertical pixel number is half of the frequency ratio between horizontal and vertical scanner while the horizontal pixel number is chose to make the pixel size match optical resolution. Usually, pixel size is half of the lateral resolution or even more which can be determined by the real sample size while each pixel is averaged by 5 to 10 data samples.

3.3 Improvement of field of view

There are series of problems if the field of view is limited. Since in general the size of the real biological samples is around 30μm to several hundred micrometers, in order to search them when the density of them is low, we have to have a field of view 10 or even more times bigger than that size. The best case is that we can get the TPEF image with the same size of the one we get from back-illuminated incoherent light. As Figure 3 shows, we add the relay imaging system which includes one scanning lens and one tube lens before the back aperture of OBJ. These two lenses act as an asymmetric 4-f system which relay the beam as well as the wavefront on the Galvano mirror and magnify the beam size to fulfill the back aperture of OBJ. Since the beam is no longer moving at the back aperture of OBJ, there is no loss for big scanning angle as long as the two lenses is big enough.

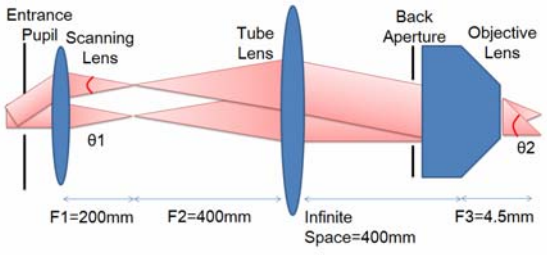
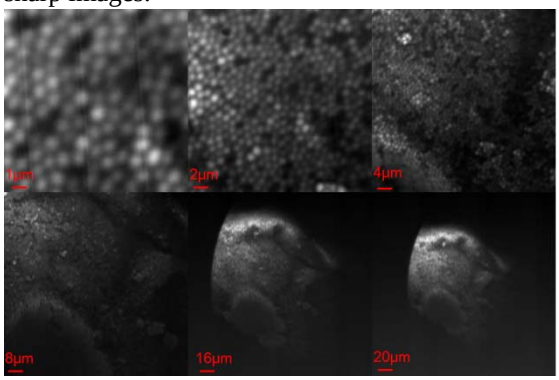


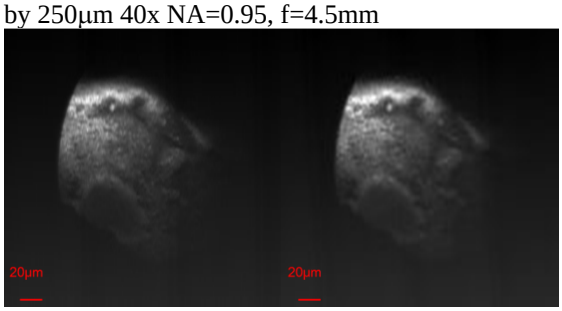
Figure 3 Relay system

Figure 4 shows the TPEF image of fluorescent beads at different field of view. When the imaging size is below 62.5μm by 62.5μm, we can clearly identify each fluorescent bead. However, when the imaging size is larger, the limitation of

data acquisition speed in horizontal direction decreases the image resolution below the optical resolution and thus we cannot resolve each single fluorescent bead. Once the imaging size is big enough, we see the sharp boundary which is due to the limitation of the size of mirrors. In Figure 4 (b), we compare the images with different horizontal pixel numbers at the same physical measuring condition. We integrate every ten horizontal pixels of the left image to get the right image. By rough seeing, there is no obvious difference in terms of image quality while if we zoom in the picture, we can see shaper textures for the left image where we don't integrate the pixels. However, if the integrated pixels' size is smaller than the optical resolution, typically, there is no difference between the two cases. But we have to use higher spatial sampling frequency which is 5 to 20 times bigger than the frequency of optical resolution in order to resolve the sharp change. In the other words, if we directly use the spatial sampling frequency same as the one of optical resolution, we can't achieve sharp images.



(a) Image size from 12.5um by 12.5um to 250um by 250μm 40x NA=0.95, f=4.5mm



(b) Left: 1600 pixels in horizontal direction; Right: 160 integrated pixels in horizontal direction

Figure 4 Improved TPEF Image

4. Conclusion

We have constructed the TPEF microscopy which can offer us an ultra-big scanning region as long as there is no blocking by the size of optics. Thus we are now trying to use mirrors and DM with large aperture. Also in order to achieve even bigger scanning region and also be easy for searching the interesting sample, we plan to add a revolver which is easy for us to switch the OBJ with different magnifications. So we can first search the TPEF image by OBJ with lower magnification and then capture fine TPEF image by OBJ with higher magnification. In terms of the TPEF image quality, the lateral resolution is less than 1 um while axial resolution is around several micrometers for a OBJ with N.A.=0.95. And the imaging rate is 0.1 to 0.2 frames per second for a sharp TPEF image.

Reference

[1] W. Denk, J.H. Strickler & W.W.Webb, Science 248, 73–76 (1990).

[2] T. Wilson & C.J.R. Sheppard, *Theory and Practice of Scanning Optical Microscopy*. Academic Press, London (1984).

[3] T.R. Corle and G.S. Kino, *Confocal Scanning Optical Microscopy and Related Imaging Systems.* Academic Press, San Deigo (1996).