Three-Photon Image-Scanning-Microscopy Enabling Deep Super-Resolution Imaging

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Laser scanning multiphoton microscopy has enabled imaging deep in highly scattering samples, as the nonlinearity causes the excitation to be strongly confined in the axial direction, that is, multiphoton microscopy comes with intrinsic sectioning of the signal. The resolution in laser scanning microscopy is given by how tightly one can focus the beam, which is the so-called Abbe limit, given by the wavelength λ, divided by twice the numerical aperture NA of the focusing element (objective). Considering the resolution improvement due to the nonlinearity and the resolution decrease due to the increased excitation wavelength, when compared to confocal microscopy, for the two- and three-photon excited microscopy the resolution worsens by a factor $\sqrt{2}$ or $\sqrt{3}$, respectively, for any given fluorophore. Several approaches have been developed for single photon excited microscopy to overcome the Abbe limit [1–5]. From those methods, structured illumination microscopy [2] and image scanning microscopy (ISM) [4] are advantageous because they can be used with virtually any fluorescent marker, which makes them the most widely applicable super-resolution methods. ISM is implemented with laser scanning confocal microscopy, and recently it was demonstrated to be applicable to two-photon microscopy [6], achieving deep imaging with a resolution better than what is achieved with confocal microscopy.

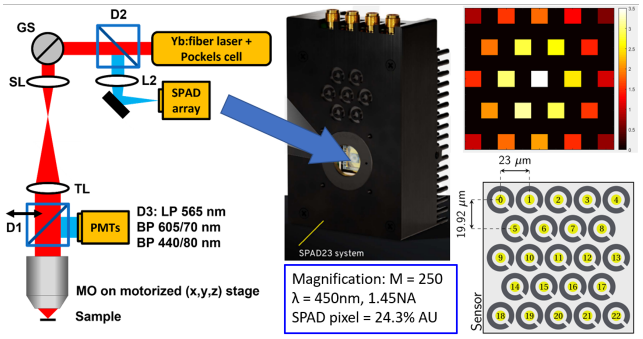

Figure 1: Experimental setup of three photon ISM, using an in-house built 4 MHz Yb-fiber laser with stretcher fiber and a 23-element SPAD array

In this work we demonstrate 3-photon excited (3PE) ISM experimentally and show the utility of this approach for deep tissue imaging. The setup is shown in Fig. 1. Using this approach, we have achieved resolution enhancements of 1.6 laterally and 1.8 axially for 3PE-ISM over standard 3PE microscopy. We demonstrate that with 3PE-ISM it is possible to surpass the resolution achievable with confocal microscopy for the same fluorophore, despite the three times longer excitation wavelength. Including deconvolution, the resolution enhancements reach factors of 2.5 in the lateral and 3.0 in the axial direction.

Finally, we use the system to image fixed biological samples in mouse spinal cord tissue stained with the DNA marker DAPI (thus highlighting the cell nuclei). The resolution and contrast enhancement offered by ISM over standard PMT detection is demonstrated in thick samples.

References

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