Technology development for deep tissue multiphoton imaging

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Abstract
Deep tissue multiphoton imaging of mouse brain using 1280-nm excitation is presented. Approximately 1-mm imaging depth is achieved in adult mice *in vivo*. Blood flow measurements at a depth of 900 μm are performed.

The three-dimensionally confined excitation volume of two-photon fluorescence microscopy (TPM) provides high resolution images from deep within turbid biological samples. TPM has been used as a standard tool for the study of blood flow, neuronal activity, and anatomy in the cortex of mouse brain. Imaging up to a depth of 600 μm for vasculature and 700 μm for neurons has been achieved with femtosecond modelocked Ti:Al_2O_3 oscillators [1]. The maximum imaging depth in TPM depends on the ability of excitation light to reach the focus unscattered (ballistic excitation photons) and emitted fluorescence to reach the detector. It scales linearly with the attenuation coefficient of the excitation light in tissue and logarithmically with average power incident on the sample surface, the duty cycle, and the collection efficiency. Theer *et al.* achieved 1 mm imaging depth using a Ti:Al_2O_3 regenerative amplifier (μJ pulses) which reduces the repetition rate from 100 MHz to 200 kHz [2]. This method sets a limit to the imaging speed and a requirement of precise synchronization of excitation pulses with image acquisition. Reduction of the pulse width is a less effective method since the width of the excitation spectrum limits the maximum spectral width of the laser pulses. A more effective strategy for improving the imaging depth is reduction of the attenuation of excitation light by tissue. As a result of the large difference between scattering mean free paths (MFP) and absorption lengths in the brain tissue, scattering is the dominant attenuation factor over water and intrinsic molecule absorption for wavelengths between 350 nm and 1300 nm. We propose using longer excitation wavelengths, specifically the 1300-nm regime, in order to reduce the effect of scattering. The use of longer excitation wavelengths will typically result in longer wavelength fluorescence emissions, where there is less absorption due to intrinsic tissue molecules, yielding an additional increase in signal collection beyond the decreased scattering of the excitation light. In this paper, the fundamental difficulties of deep tissue imaging will be discussed. We then compare the maximal TPM imaging depth achieved with 775-nm excitation to that achieved with 1280-nm excitation using *in vivo* and *ex vivo* TPM of vasculature in the cortex of adult mouse brain. Approximately 1-mm imaging depth can be achieved in *in vivo* imaging of adult mouse at 1280 nm with ~ 1 nJ pulse energy (Fig. 1). We also record blood flow speed in individual vessels at depths of up to 900 μm. Several Practical issues of long wavelength TPM will be discussed. Measurement of two-photon excitation cross sections of several long wavelength fluorescent probes will be presented. The main challenge for the long wavelength TPM is the complexity and cost of the excitation source. A promising new femtosecond fiber source for long wavelength TPM will be briefly discussed.

References
1. Helmchen, F. and Denk,W., "Deep tissue two-photon microscopy," Nature Methods 2(12), 932-940 (2005).
2. Theer, P., Hasan, M. T. and Denk, W., "Two-photon imaging to a depth of 1000 μm in living brains by use of a Ti:Al2O3 regenerative amplifier," Opt. Lett. 28, 1022-1024 (2003).

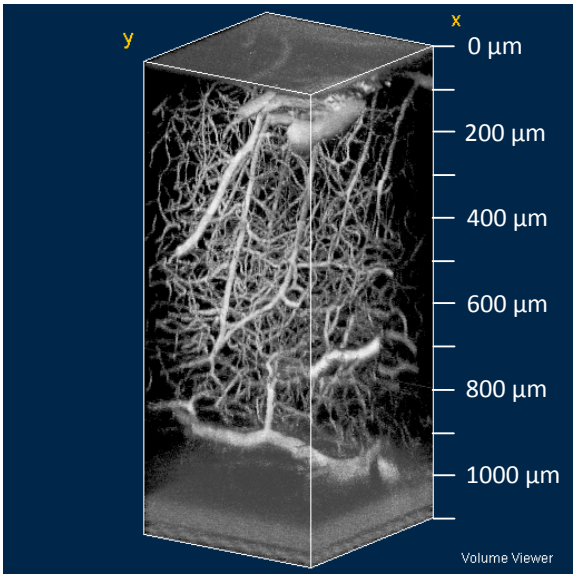

Figure 1: 3D re-construction of the cortical vasculature of an adult mouse imaged *in vivo*. An imaging depth of 1000 μm is achieved.