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Mutlicolor two-photon excitation for increasing fluorescence excitation depth

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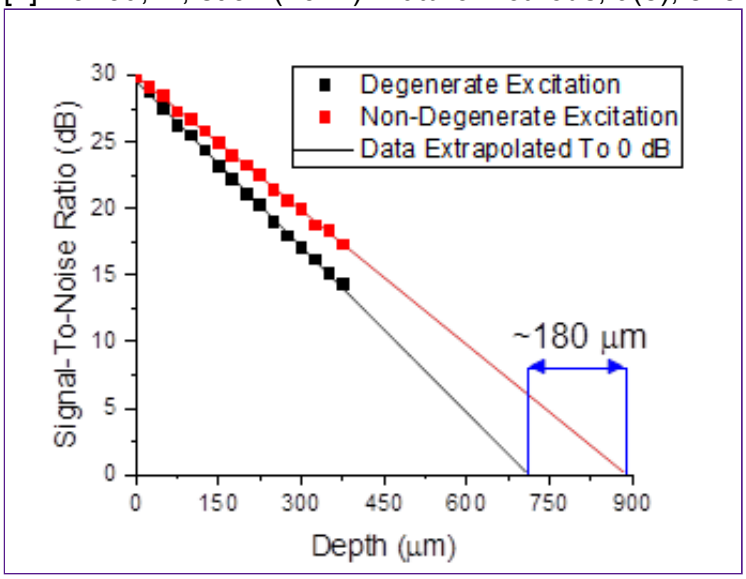
Abstract:
The advent of 2-photon microscopy has enabled *in vivo* imaging of cerebrocortical structure and function with micron resolution. Yet, penetrating deep into the cortex remains a challenge due to the scattering and absorption of light by cerebral tissue. The combined unwanted effect of scattering and absorption can be reduced by employing longer illumination wavelengths in the range of 1300-1700 nm. This range, however, is not suitable for conventional, a.k.a. “degenerate” 2-photon excitation (D-2PE) of visible probes (e.g., with red or green emission). Here, we address this problem by multicolor 2PE, a.k.a. non-degenerate 2PE (ND-2PE). The principle of multicolor 2-photon imaging has been demonstrated *in vivo* for simultaneous excitation of multiple chromophores [1]. We are adapting this technology for deep imaging.
We excite the fluorophores via the simultaneous absorption of two photons of different energy (i.e., wavelength) using two different pulsed lasers sources. The lasers are spatially aligned and temporally synchronized. One laser is tuned to the near infrared (NIR, 700-900 nm), the second laser is tuned to the infrared (IR, 1300-1700nm) to match the energy needed for excitation of the fluorophore. The IR laser is used to compensate for NIR power loss due to tissue scattering. Furthermore, IR wavelengths are scattered less by tissue than NIR, allowing for deeper penetration into the cortex.
We excited fluorescein (FITC) submerged within an intralipid suspension of varying

concentration. Excitation was provided by a pulsed Ti:Sapphire laser tuned to 825nm and an optical parametric oscillator tuned to 1315nm. Under these conditions, we can compare the D-2PE and ND-2PE fluorescence. We find that ND-2PE fluorescence decreases over a longer distance within the intralipid as compared to D-2PE fluorescence for all concentrations. These results are summarized in the attached figure. Thus, we have demonstrated a proof-of-principle for increased fluorescence excitation depth using non-degenerate excitation.

[1] Mahou, P., et al. (2012). *Nature Methods*, *9*(8), 815-818.



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