# Overcoming the fundamental limit of two-photon microscopy with non-degenerate excitation

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**Abstract:** We use non-degenerate two-photon excitation where the two excitation beams are displaced in space outside the focal volume to increase the signal-to-background ratio (SBR), overcoming the fundamental penetration depth limit of conventional two-photon microscopy. 

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## 1. Introduction

Penetration depth of two-photon microscopy (TPM) is ultimately limited by out-of-focus excitation (OFE) [1] (Fig, 1 (a)). In conventional TPM, two photons required for excitation are derived from the same laser source and have the same energy. This is called degenerate two-photon excitation (D-TPE). In non-degenerate two-photon excitation (ND-TPE), the two photons are derived from two synchronized pulsed laser sources generating photons of different wavelength. Recently, we have demonstrated that the probability of ND-TPE is higher compared to D-TPE [2, 3]. In the present study, we show that non-degenerate two-photon microscopy (ND-TPM) also has the potential to significantly decrease OFE by spatially displacing the two laser beams such that they overlap only in the focal spot [4] (Fig, 1 (a)). This effect depends on several parameters including degree of the spatial separation of two beams, quality of the beam overlap in the focal volume, and point-spread function (PSF) for each beam. Here we investigate key parameters important for achieving the optimal SBR in ND-TPM.

## 2. Methods

As shown in Fig. 1(b), the primary light source in our ND-TPM was a double output femtosecond pulsed laser (Chameleon Discovery, Coherent) with 80 MHz repetition rate with one beam fixed at 1040 nm and a second tunable beam ($660-1320$ nm). The path length difference between the two beams was compensated for using a custom optical delay line in one of the beam paths.The beams entered the back aperture of the objective side-by-side, parallel to each other and the optical axis (Fig. 1(b)). The two beams were overlapped in the focal plane using steering mirrors and an automated defocuser. To scan the two spatially displaced beams we employed 10-mm Galvo mirrors. The Galvo mirrors were imaged to the back aperture of the objective using a scan lens and a tube lens that formed a 4f system. An achromatic objective lens (XLUMPLFLN20XW, NA=1) focused the light to the sample and collected the emitted fluorescence from the sample. The fluorescence emission was detected via a photomultiplier tube.

## 3. Results

First, we performed diagnostics of our optical system by measuring PSF for each beam (1040 nm and 1300 nm), and their overlap (Fig. 1(c)). The PSF measurements were achieved by imaging 1-$\mu$m red fluorescent beads in 3D using (i) D-TPE produced by each laser beam in isolation (the beads exhibited small but non-zero absorption cross-section at both laser wavelengths) and (ii) ND-TPE produced by the two beams combined (Fig. 1(c)). ND-TPE signal was calculated by subtracting the D-TPE generated by each individual beam from the total detected signal. As expected, the individual PSFs were tilted with respect to the optical axis and partially overlapped in the focal volume generating ND-TPE. We imaged a tissue-like phantom composed of 0.5% agarose gel and 1-$\mu$m red fluorescent beads, while varying the distance between the beams from colinear (full overlap) to side-by-side (zero overlap) configurations. The images shown in Fig. 1(d) were taken 700 $\mu$m below the surface of the phantom. Specifically, we observed higher SBR with the side-by-side configuration compared to the colinear configuration

(Fig. 1(e)), with approximately 6 times enhancement in SBR. The amount of enhancement in SBR depended on the amount of displacement, the beam size (effective numerical aperture (NA)), and the quality of the beam overlap in the focal volume. We used our recently developed beam propagation simulation technique [5] to characterize the effects of the mentioned parameters on SBR. Based on our simulation results, both the efficiency of the ND-TPE (signal) and OFE (background) decreased while increasing the spatial separation of the beams. However, the rate of decrease in the background was higher, compared to that of the signal. Therefore, the overall SBR increased by increasing the beam displacement (Fig. 1(e)). We also studied the effect of the beams size (effective NA) on SBR (Fig. 1(f) and observed that for the optimal selection of the beam displacement and effective NA we can achieve around 60 times higher SBR compared to the colinear configuration that would translate into 4 scattering lengths implying that we increase the depth penetration by $>$ 400 microns.

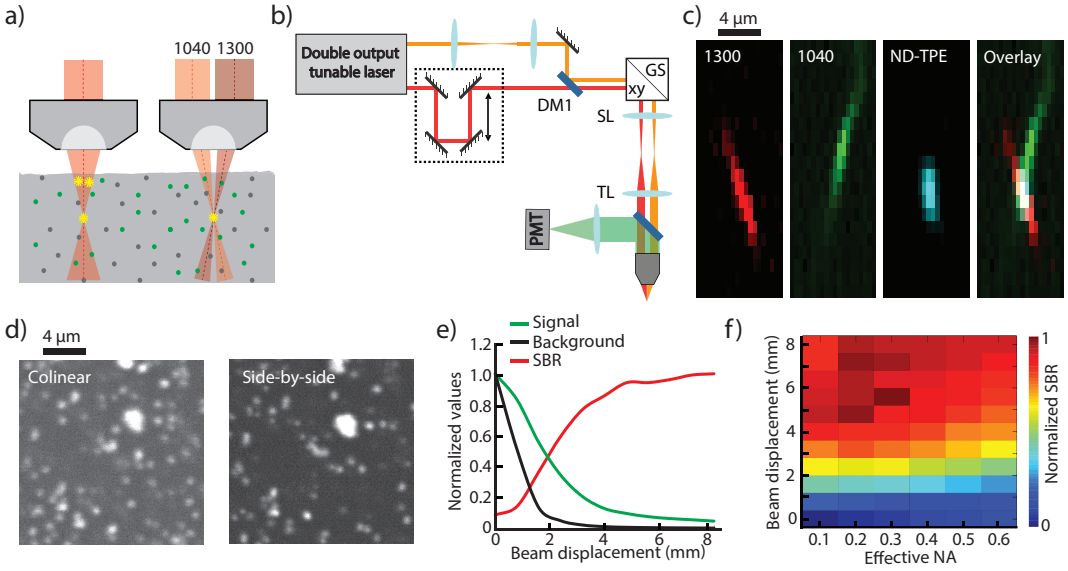


Fig. 1. Increased SBR with displaced beams in ND-TPM. **a)** Left: D-TPE excites the sample both near the surface and within the focal volume. Right: by selecting the two excitation beams for ND-TPE outside of the excitation spectrum of the flurophore and spatially displacing the beams, the sample is excited only in the focal volume where the two beams overlap. **b)** Simplified schematic of ND-TPM setup: GS-XY galvanometer scanner, SL-Scan lens, TL-Tube lens, DM - Dichroic mirror, OL -objective lens, PMT - photomultiplier tube. **c)** PSFs of individual beams (1040 nm and 1300 nm) and ND-TPE. The last panel shows the overlap of the PSFs. **d)** Images of bead phantom with colinear and side-by-side configurations of the ND-TPE. **e)** Simulation results for the normalized values of signal, background, and SBR versus the beam displacement. **f)** Simulation results for normalized SBR versus effective NA and beam displacement.

## 4. Conclusions

Here we showed that the ND-TPM is capable of overcoming OFE which is the ultimate limiting factor for penetration depth in conventional TPM. We predict that under optimal conditions ND-TPM would increase the penetration depth in brain tissue by $>$ 400 microns compared to conventional D-TPM.

## References


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