Multicolor two-photon excitation for increasing fluorescence excitation depth

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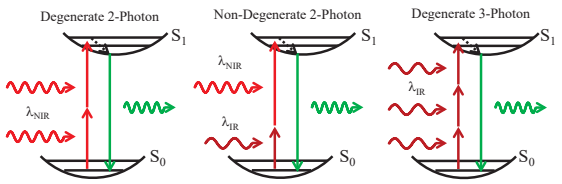
SUMMARY

The maximum imaging depth for standard 2-photon fluorescence microscopy in brain tissue is limited by several factors including light absorption and scattering and background fluorescence excitation, a.k.a. "out-of-focus" excitation, at the brain surface. Here we address these limitations by implementing non-degenerate 2-photon excitation (ND-2PE) using two synchronized, pulsed femtosecond laser beams of different wavelengths (color) [1]. The first beam is tuned within the standard near infrared (NIR) range of excitation wavelengths used in degenerate 2-photon microscopy. The second laser is tuned to infrared (IR) wavelengths used in 3-photon excitation [2]. The presence of two laser beams allows:

(1) tuning the combination of wavelengths to **optimize excitation cross section for each fluorophore**;
(2) independent control of power (and polarization) such that an increase in the **IR power can be used to compensate for the loss of NIR power due to scattering and absorption**;
(3) spatial displacement of the two beams to **limit the overlap to the focal volume, minimizing background excitation**.

BACKGROUND



$I_f \propto I_{NIR}^2 \sigma_{D2PE}$ $I_f \propto I_{NIR} I_{IR} \sigma_{ND2PE}$ $I_f \propto I_{IR}^3 \sigma_{3PE}$

Fig. 1. Jablonski diagram for D-2PE , ND-2PE, and 3PE.

Under ND-2PE, the total fluorescence intensity I_f can be modelled by the Beer-Lambert law type attenuation process:

$$I_f = \left(\{I_{NIR}^2 \sigma_D [D] e^{-2z/\alpha_{NIR}}\} + \left\{I_{NIR} I_{IR} \sigma_{ND} [D] e^{-z/(\alpha_{NIR}^{-1} + \alpha_{IR}^{-1})}\right\}\right) \beta e^{-z/\alpha_f}$$

I - fluorescence intensity
σ - absorption cross section
ϕ - quantum yield
α - attenuation length for a specific λ
β - imaging geometry factor
$[D]$ - fluorophore concentration
z - imaging depth

EXPERIMENTAL SETUP

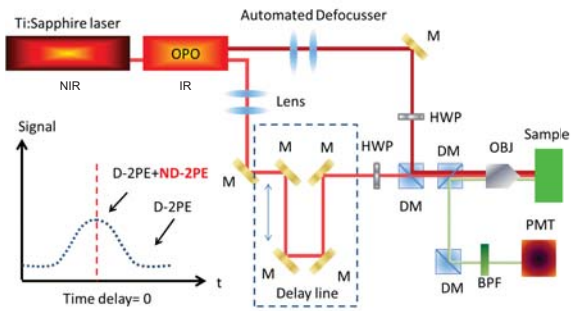


Fig. 2. Experimental setup for demonstration of ND-2PE. HWP, half wave plate; DM, dichroic mirror; OBJ, microscope objective; BPF, band pass filter; PMT, photomultiplier. M, mirror.

REFERENCES

[1] Mahou, P., et al. (2012). *Nature Methods*, 9(8), 815818; [2] Horton, N.G., et al. (2013). *Nature Photonics* 7, 205–209; [3] Kobat, D. et al. (2008). *Biomedical Optics*, p. BMF6; [4] Wang C., et al. (2008). *JOSA B* 25, 976–982.

SPECTROSCOPY: EXCITATION EFFICIENCY

$$\langle F \rangle_{ND} = \frac{\sqrt{\pi}\,(\eta_2 \delta)\,\phi\, C\, \lambda_{pump}\, \lambda_{probe}\, \langle P \rangle_{pump}\, \langle P \rangle_{probe}}{2\, h^2\, c^2\, f_{rep}\, \sigma_X \sqrt{\lambda_{pump}^2 + \lambda_{probe}^2}} \exp\left(\frac{-\tau^2}{2\sigma_X^2}\right)$$

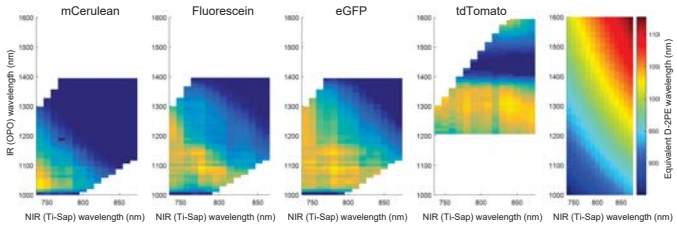


Fig. 3. Cross section measurements. Left: ND-2PE cross section for 4 different fluorophores. Right: the equivalent D-2PE wavelengths.

IMAGING: PENETRATION DEPTH

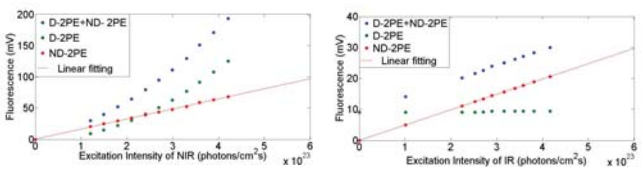


Fig. 4. Dependence of fluorescence intensity on power of NIR and IR [3]. (a) Dependence on NIR power (IR power is fixed at 4.16×10^{23} photons/cm²s). (b) Dependence on IR power (NIR power is fixed at 1.18×10^{23} photons/cm²s).

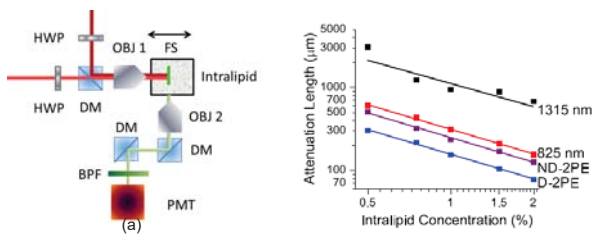


Fig. 5. Attenuation in scattering medium. (a) Experimental setup. (b) Attenuation length as a function of intralipid concentration.

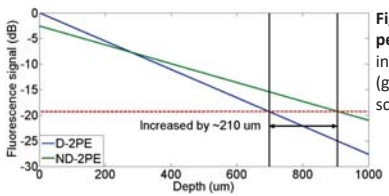


Fig. 6. Theoretical analysis of the penetration depth. Fluorescence intensity under D-2PE (blue) and ND-2PE (green) as a function of depth in the scattering medium.

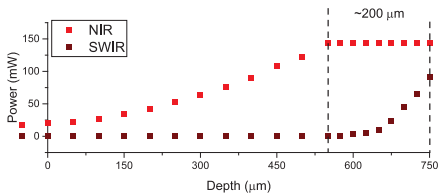


Fig. 7. Increase in penetration depth via ND-2PE.

1. With NIR alone: NIR power is increased with depth to compensate for scattering.
2. After NIR power is exhausted, an increase in IR power allows deeper imaging.

IMAGING: RESOLUTION

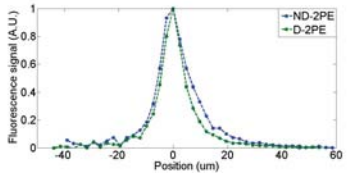


Fig. 8. Axial resolution with 0.55-NA objective lens.

IMAGING: OUT-OF-FOCUS EXCITATION

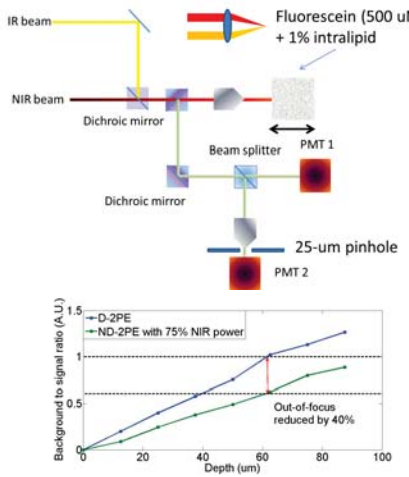


Fig. 9. Excitation with spatially displaced NIR and IR beams. Top: experimental setup . Bottom: signal to background ratio at different depths in scattering medium.

CONCLUSIONS

1. The choice of NIR and IR wavelength must take into account (i) fluorophore-specific ND-2PE cross section and (ii) attenuation of each wavelength.
2. Increasing the photon flux of the IR beam helps to compensate for the scattered loss of the NIR beam.
3. ND-2PE provides greater penetration depth in the scattering medium.
4. Axial resolution can be improved by spatial displacement of the beams [3].
5. Beam displacement also helps reducing out-of-focus excitation [4].
6. Adaptive optics would further improve penetration depth.

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