Imaging depth limit analysis in multiphoton microscopy using the beam propagation method

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Abstract: We apply our recently developed beam propagation model to simulate wave propagation in scattering biological tissue. The imaging depth limit is estimated for two-photon, three-photon, and non-degenerate two-photon microscopy.

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Multiphoton microscopy is a major tool used for *in vivo* deep tissue optical imaging that can resolve structural and functional information on the cellular level. Compared to techniques utilizing single-photon excitation of fluorophores, longer wavelengths are commonly used in multiphoton microscopy with larger scattering mean free paths which facilitate deep penetration. More importantly, non-linear excitation, including 2-photon (2P) and 3-photon (3P) excitation, can significantly enhance the signal to background ratio (SBR). Recently, a new technique that uses two beams of different wavelengths for 2P excitation, namely non-degenerate 2P (ND-2P) microscopy, has demonstrated the potential of improving SBR against degenerate 2P excitation, while still maintaining high excitation efficiency compared to 3P excitation [1]. However, scattering is still a main issue in multiphoton microscopy, which degrades both the SBR and the resolution governed by the quality of the point spread function (PSF). The evaluation of the performance of various multiphoton microscopy systems under different imaging conditions is of great interest, which will guide experimental designs.

To quantitatively analyze the fundamental imaging depth limit in 2P, 3P, and ND-2P microscopy for any imaging system and biological sample, we have developed a numerical model based on the beam propagation method that computes wave propagation in scattering media [2]. The scattering biological medium is modeled as layers of phase masks that distort the phase profile of the propagating wavefront. Unlike many other studies where the beam propagation method is used as a heuristic model [3], our method has explicitly established the relation between the microscopic parameters σ_p and σ_x of the phase masks, as explained in Fig.1 (a)-(d), with the scattering mean free path ℓ_s, anisotropy factor g, the pixel size a and the phase mask layer distance d. The input wavefront geometry such as the numerical aperture (NA) of the objective, the input Gaussian beam, and the wavelength λ can also be adjusted. The wave-optic simulation tool is able to capture small scale features ($\sim\lambda$) such as speckle patterns induced by wave interference effects. These are not modeled in the existing particle-picture based simulations, including Monte-Carlo simulations and a pioneering analytical study of the imaging depth limit in 2P microscopy [4].

Using this beam propagation model, we have estimated the SBRs as functions of depths for the three types of multiphoton microscopy mentioned above with examples shown in Fig. 1. The scattering sample used is the same for the three cases with $\ell_s = 100\,\mu m$ for $\lambda = 1300\,nm$ and anisotropy factor $g = 0.9$. The SBR is calculated with the signal and background intensity obtained with the total fluorescence energy integrated over the focal volume and out-of-focus volume respectively [4]. The imaging depth limit z_l is estimated at SBR=1. Note that for ND-2P microscopy, the arrangement of the two beams is crucial for the imaging quality. In the example of Fig. 1 (l)-(n), the two Gaussian beams both have NA=0.3 and are placed side-by-side. We will use our model to further explore the configuration of the two beams for optimized performance of ND-2P

microscopy. The SBR calculation is dependent on the dye concentration used in experiments and the focal volume used in calculations to separate the signal and background fluorescence intensity. We have not taken into account the variation of the focal volume in the presence of scattering yet, which we will explore in the future. This could result in the estimated imaging depth limit smaller than expected, since the focal volume should scale with the size of the PSF which increases with depth. We will also quantify the degradation of the resolution in terms of the broadening of the point spread function for various types of imaging systems as illustrated in [2].

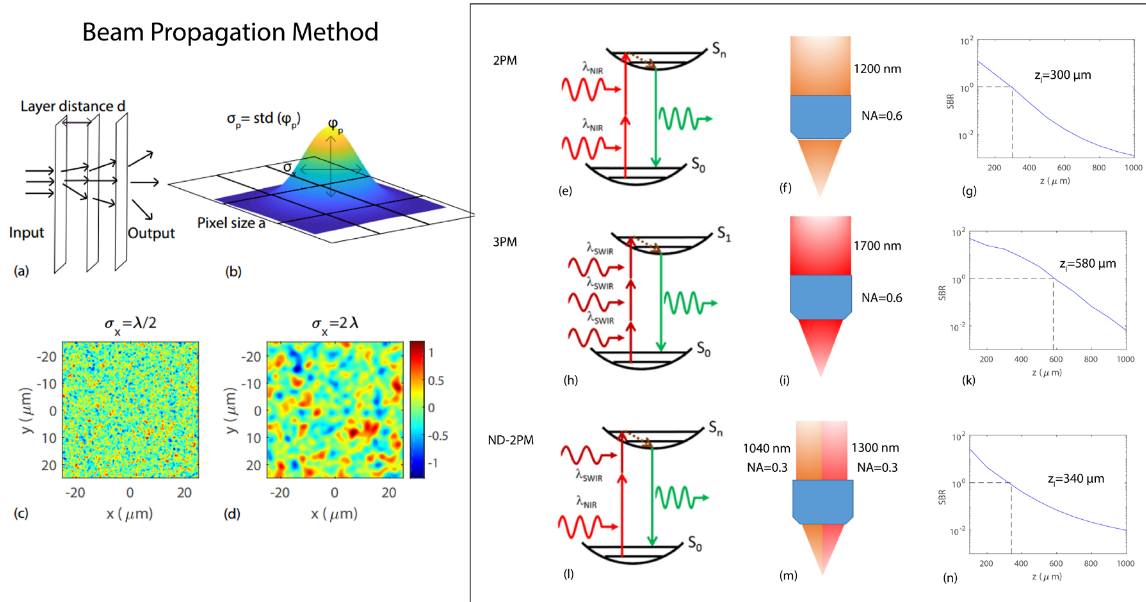

Fig.1. (a) Illustration of the beam propagation method. (b) The parameters of the phase masks. σ_p is the standard deviation of the seed phase φ_p at each pixel. The seed profile is convolved with a spatial Gaussian profile with width σ_x. (c)-(d) Examples of phase masks with $\sigma_p = \pi/10$, $\lambda = 500\ nm$, pixel size $a = \lambda/4$, $\sigma_x = \lambda/2$ (c), and , $\sigma_x = 2\lambda$ (d). Illustrations and SBR calculations obtained with the beam propagation method for 2P (e)-(g), 3P (h)-(k) and ND-2P (l)-(n) microscopy with NA of the objective fixed to be 0.6. The scattering mean free path $\ell_s = 100\ \mu m$ for $\lambda = 1300\ nm$ and anisotropy factor $g = 0.9$. The dye concentration used is 0.04 of the total volume. The wavelength dependence of ℓ_s is $\frac{\ell_{s1}}{\ell_{s2}} = (\frac{\lambda_1}{\lambda_2})^{1.2}$ as for brain tissue [5]. The imaging depth limit z_l is estimated at SBR=1.

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