Reduced effects of spherical aberration on penetration depth under two-photon excitation

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We compare the effects of spherical aberration on the penetration depth of single-photon and two-photon excitation for instances in which the aberration is caused by the refractive-index mismatch when a beam is focused through an interface. It is shown both theoretically and experimentally that two-photon fluorescence imaging experiences less spherical aberration and can thus propagate to a deeper depth within a thick medium. © 2000 Optical Society of America

OCIS codes: 180.1790, 180.2520, 180.6900.

Two-photon (2-p) fluorescence microscopy offers numerous advantages over single-photon (1-p) fluorescence microscopy.[1] These advantages originate from two physical aspects, the nonlinear dependence of the fluorescence intensity and the illumination wavelength. The nonlinear dependence of the fluorescence intensity on the illumination power under 2-p excitation results in a pinpoint excitation or emission nature and then confines photobleaching and photodamaging to the focal region of an objective. If an infrared laser beam is used for 2-p excitation, the Rayleigh scattering effect is significantly reduced in comparison with 1-p excitation at a visible wavelength, because the strength of this type scattering is inversely proportional to the fourth power of the illumination wavelength. In this paper, we show that the use of an infrared wavelength for 2-p excitation also provides an advantage of reducing aberration. In particular, spherical aberration caused by the refractive-index mismatch at an interface under 2-p excitation affects the penetration depth less significantly than that under 1-p excitation.

When a wave is focused by a lens into an interface of mismatched refractive indices n_1 and n_2, its wave front becomes distorted. Figure 1 shows that incident angles for the two shown rays are slightly different, resulting in rays being focused at different positions along the optical axis, i.e., giving rise to spherical aberration. This aberration function depends on the focus depth d, the refractive indices, the excitation wavelength, and the angle of convergence of a ray and can be written as[2–4]

$$k_0\Phi(\theta_1, \theta_2, -d) = -k_0 d(n_1 \cos\theta_1 - n_2 \cos\theta_2), \quad (1)$$

where θ_1 and θ_2 denote the angles of incidence and refraction, respectively, and are linked by Snell's law. Thus the effect of the aberration becomes significant, especially at deep depths. The wave number k_0 is equal to $2\pi/\lambda$. It is therefore clear that for a given depth d 2-p excitation experiences less aberration than 1-p excitation owing to the longer excitation wavelength in the former case.

The three-dimensional (3-D) intensity point-spread function for the lens at a point $\mathbf{r}_p(r, \phi, z)$ in the second medium is given, in cylindrical coordinates, by

$$I(r, \phi, z) = |I_0|^2 + 4|I_1|^2 \cos^2\phi + |I_2|^2 + 2\cos(2\phi)\mathrm{Re}(I_0 I_2^*). \quad (2)$$

Here the integrals I_0, I_1, and I_2 are given by Török *et al.*[2] and include the contribution from Eq. (1). The 3-D intensity point-spread function for 1-p confocal fluorescence microscopy and 2-p fluorescence microscopy (no pinhole) can be expressed, respectively, as[5–7]

$$I_{1\text{-p}}(r, \phi, z) = I_{\text{ex}}(r, \phi, z) I_f(r, \phi, z), \quad (3)$$

$$I_{2\text{-p}}(r, \phi, z) = I_{\text{ex}}^2(r, \phi, z). \quad (4)$$

Here $I_{\text{ex}}(r, \phi, z)$ and $I_f(r, \phi, z)$ are given by Eq. (2) for excitation and fluorescence wavelengths, respectively.

Although Eqs. (3) and (4) have been calculated

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Received 4 February 2000; revised manuscript received 9 May 2000.

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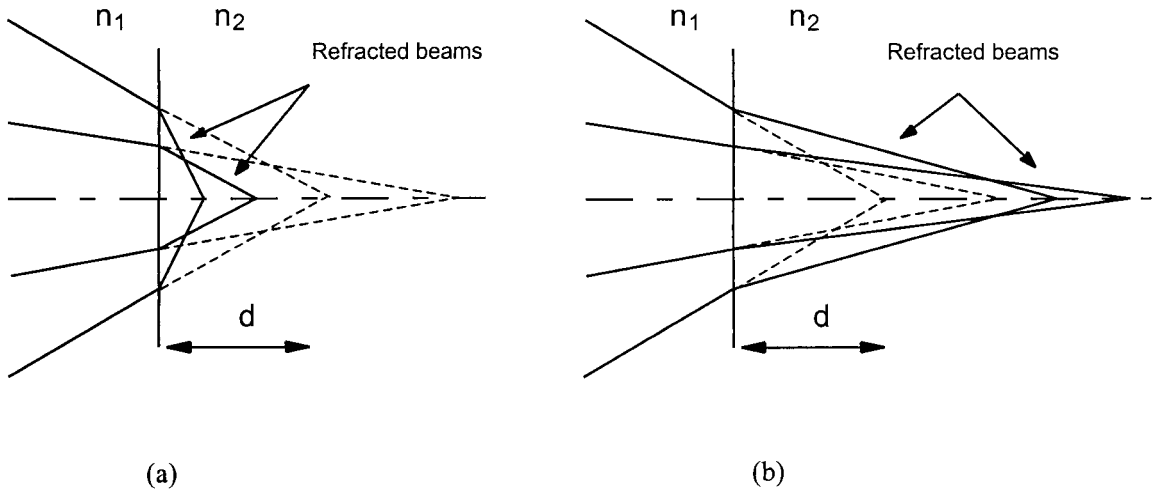

Fig. 1. Schematic diagram of beams being refracted on an interface between two media: (a) $n_1 > n_2$ and (b) $n_1 < n_2$.

before,[5–7] it is difficult to conduct experiments to measure them. However, the axial response to a thick fluorescent material can be easily measured and has been used as a measure of penetration depth.[7,8]

For 1-p excitation, the axial response to a thick fluorescent material at a depth d is[9]

$$I(d) = \int_{-d}^{\infty} \int_{0}^{2\pi} \int_{0}^{\infty} I_{1\text{-p}}(r, \phi, z) r \mathrm{d}r \mathrm{d}\phi \mathrm{d}z. \qquad (5)$$

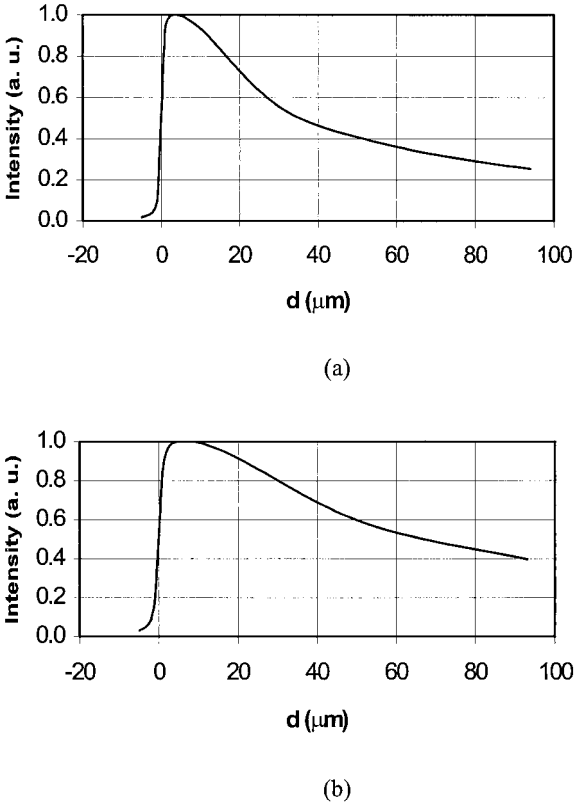

Fig. 2. Calculated fluorescence axial response to a thick polymer (a) under 1-photon and (b) under 2-photon excitation.

When 2-p excitation is used, the axial response is given by the same expression as Eq. (5), with $I_{1\text{-p}}$ replaced with $I_{2\text{-p}}$.

To investigate the axial response, we take as an example the fluorescent polymer of refractive index 1.48 (Ref. 7), immersed in air of refractive index 1.0. The polymer can emit fluorescence at a wavelength of 520 nm under 1-p (wavelength 488 nm) and 2-p (wavelength 800 nm) excitation.

The calculated fluorescence axial responses under 1-p and 2-p excitation are shown in Fig. 2. It can be seen that the 1-p fluorescence signal strength is degraded as the focus depth d is increased. At $d = 40$

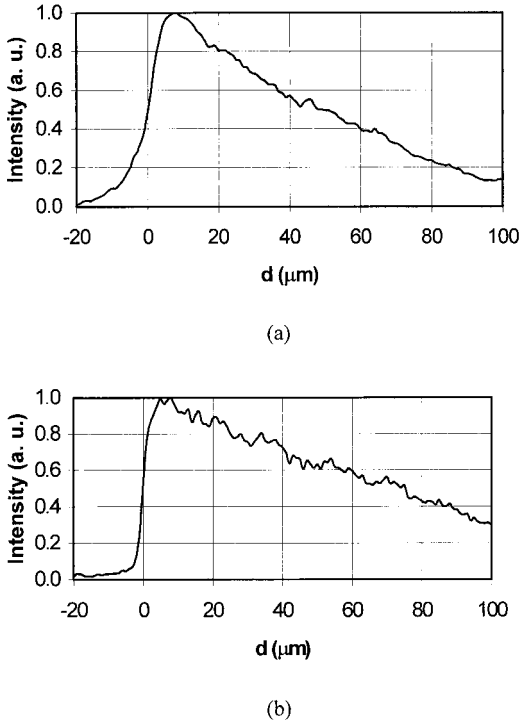

Fig. 3. Measured fluorescence axial response to a thick polymer (a) under 1-photon and (b) under 2-photon excitation.

μm, the 1-p fluorescence intensity is halved. The depth at which the 2-p fluorescence intensity drops to half of its maximum is approximately 70 μm, which increases the penetration depth by 75% compared with that under 1-p excitation.

To confirm the calculated results, we used a reflection-mode fluorescence microscope incorporating a Ti:sapphire pulsed laser (Spectra-Physics: Tsunami).[6] An infinitely corrected Olympus dry objective (ULWD MSPlan 80 ∞/0 N.A. = 0.7) was used for imaging. A 2-p excitation was achieved with a wavelength of 800 nm from the pulsed laser, whereas 1-p excitation was achieved with a wavelength of 400 nm that was produced by a frequency-doubled unit operating at a wavelength of 800 nm.

Figure 3 shows the experimental 1-p and 2-p fluorescence axial responses, which agrees well with the theoretical prediction. It is confirmed that 2-p excitation experiences less spherical aberration caused by the refractive-index mismatch and can propagate to deeper depths within a thick medium than 1-p excitation. This advantage results from the utilization of a longer wavelength for illumination and is of importance to 3-D fluorescence imaging[1] or 3-D optical data storage.[7] In general, 2-p excitation can reduce the effect of any aberration, but the amount of the improvement depends on the distribution of aberration.

The authors acknowledge support from the Australian Research Council. This research was part of the honors thesis completed by the D. Ganic at Victoria University.

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