Refractive-index-induced aberrations in two-photon confocal fluorescence microscopy

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Summary
The effect of refractive index mismatch on the image quality in two-photon confocal fluorescence microscopy is investigated by experiment and numerical calculations. The results show a strong decrease in the image brightness using high-aperture objectives when the image plane is moved deeper into the sample. When exciting at 740 nm and recording the fluorescence around 460 nm in a glycerol-mounted sample using a lens of a numerical aperture of 1.4 (oil immersion), a 25% decrease in the intensity is observed at a depth of 9 µm. In an aqueous sample, the same decrease is observed at a depth of 3 µm. By reducing the numerical aperture to 1.0, the intensity decrease can be avoided at the expense of the overall resolution and signal intensity. The experiments are compared with the predictions of a theory that takes into account the vectorial character of light and the refraction of the wavefronts according to Fermat’s principle. Advice is given concerning how the effects can be taken into account in practice.

Introduction
Recently, two-photon excitation has emerged as a new powerful technique in fluorescence microscopy (Denk et al., 1990). Two-photon excitation depends quadratically on the intensity at focus. The major advantages of two-photon excitation microscopy result from its quadratic behavior: fluorescence emission stems only from the focus, thus allowing for three-dimensional imaging, and bleaching is confined to the focal region. Further, UV-absorbing dyes can be excited by red or infrared light. A drawback of two-photon excitation microscopy is a decreased axial resolution due to the long excitation wavelength. This can be overcome at the expense of signal intensity by applying confocal detection (Hell & Stelzer, 1992; Sheppard & Gu, 1992).

In confocal systems it has been observed that the refractive index mismatch between the immersion system and the specimen results in a loss of image intensity and resolution (Rigaut et al., 1990; Carlsson, 1991; Shaw & Rawlins, 1991). Furthermore, the object is incorrectly scaled along the optical axis (Carlsson, 1991; Visser et al., 1992). These effects have been described as intrinsic consequences of the confocal set-up (Hell et al., 1993). Due to the growing interest in two-photon confocal fluorescence microscopy, it is interesting to investigate the effects in this technique.

Theory
For a confocal microscope, the point spread function, i.e. the image $h_{conf}$ of a point-like object, is given by the product of the illumination point spread function, $h_{ill}$, and the detection function, $h_{det}$:

$$h_{conf}(x, y, z) = h_{ill}(x, y, z) \times h_{det}(x, y, z)$$

where $(x,y,z)$ are the coordinates of a point in the focal region. For two-photon excitation, two photons have to be absorbed simultaneously by the fluorophore. Their wavelength is twice the wavelength needed for one-photon excitation. Because it depends on two photons, the excitation point spread function for this process, $h_{2ph_{exc}}$, is given by the square of the one-photon point spread function, $h(x,y,z, \lambda)$ at the wavelength $\lambda_{exc}$ (Sheppard & Gu, 1990):

$$h_{2ph_{exc}} = h_{ill}^2(x, y, z, \lambda_{exc})$$

With a fluorescence wavelength $\lambda_{fluor}$, the two-photon confocal point spread function can be written as

$$h_{2ph_{conf}}(x, y, z, \lambda_{exc}, \lambda_{fluor}) = h_{ill}^2(x, y, z, \lambda_{exc}) \times h_{det}(x, y, z, \lambda_{fluor}).$$

The point spread functions $h_{ill}$ and $h_{det}$ are evaluated using the following expression taking the influence of a refractive
index step into account (Hell et al., 1993):

\[
h(x, y, z) = \left( \begin{array}{c}
E_x(x, y, z) \\
E_y(x, y, z) \\
E_z(x, y, z)
\end{array} \right) = \text{const} \int_0^{2\pi} \int_0^\pi \sqrt{\cos \theta_1 \sin \theta_1} \\
\left[ \tau_\phi \cos (\phi - \phi_0) \cos \theta_2 \sin \phi + \tau_\psi \sin (\phi - \phi_0) \sin \phi \right] \\
\times \left[ \tau_\phi \cos (\phi - \phi_0) \cos \theta_2 \sin \phi - \tau_\psi \sin (\phi - \phi_0) \cos \phi \right] \\
\tau_\phi \cos (\phi - \phi_0) \sin \theta_2 \\
\times \exp(i k_1 s_1 + i k_2 s_2) d\phi_1, d\phi_2
\]

with \( k_1 = 2\pi n_1/\lambda \) and \( k_2 = 2\pi n_2/\lambda \), where \( n_1 \) and \( n_2 \) are the refractive indices of immersion system and sample, respectively. \( \theta_1 \) denotes the polar angle of the light cone, \( \alpha \) its maximal value, i.e. the semi-angle of the aperture, and \( \theta_2 \) the refractive angle. \( \phi \) is the azimuth angle of the light cone and \( \phi_0 \) the polarization angle of the incoming electric field.

The electrical field vector \( (E_x, E_y, E_z) \) in the focal region is calculated according to a Huygens–Fresnel construction. This corresponds to integrating the contributions of secondary wavelets from each point of the spherical wavefront in the objective exit pupil. The amplitude of the secondary wavelets varies with the polar angle \( \theta_1 \) by a factor \( \cos(\theta_1)^5 \), because the spherical wave originates from a plane wave illuminating the objective entrance pupil (Richards & Wolf, 1959). For the isotropically emitted fluorescence light this factor has to be omitted.

The light travelling towards the focus is refracted at the interface between immersion system and sample. The transmission of its s- and p-polarized components is described by the coefficients \( \tau_\phi \) and \( \tau_\psi \), respectively. They are given by Fresnel’s equations (Born & Wolf, 1980, pp. 39–41). But more important than this amplitude variation is the optical path through the refracting interface, because its length determines the phase of the interfering wavelets. The path can be split up into two parts, \( s_1 \) with the angle of incidence \( \theta_1 \) in the first medium, and \( s_2 \) with the refractive angle \( \theta_2 \) in the second medium. They have to be calculated numerically. This approach to calculating the aberrated point spread function has the advantage that it is valid for all numerical apertures.

Materials and methods

To investigate the influence of refractive index mismatch on the imaging, axial responses to a layer of the dye dispersed in a solvent were recorded. This method is suited for comparison of the experiment with theory.

In a sea with a homogeneous distribution of fluorophores, each illuminated point emits fluorescent light. Therefore, to calculate the response at a certain position \( z_{\text{NFP}} \) of the nominal focus the contributions of all points \((x, y, z)\) in the sample have to be integrated:

\[
I_{\text{obs}}(z_{\text{NFP}}, x, y, z, \lambda_{\text{exc}}) = \int \int \int h^2(z_{\text{NFP}}, x, y, z, \lambda_{\text{exc}}) \\
\times h(z_{\text{NFP}}, x, y, z, \lambda_{\text{fluor}}) dx dy dz
\]

The optical path through the refractive interface was minimized numerically by means of Newton’s algorithm. The calculations were carried out on a workstation.

Experimentally, the responses are obtained by scanning the sample in the axial direction through the focus and recording the signal intensity and the stage position. The experiments were carried out with the UV-dye Coumarin 138 (Eastman Kodak) with maximal absorption at 365 nm and maximal fluorescence at 447 nm. The samples were prepared by solving the dye in two solvents with different refractive indices. Glycerol was chosen as a frequently employed mountant. The refractive index of the Coumarin:glycerol solution was measured with a refractometer to be 1.47. Water was chosen as a second example because it is, in practice, the substance with the largest refractive index mismatch compared with the refractive index of 1.518 for the immersion oil. The refractive index of the Coumarin:water solution was 1.33. The solution was mounted between a cover glass and a microscope slide. The resulting sample thicknesses were about 4 \( \mu m \) for water and 9 \( \mu m \) for glycerol.

The two-photon excitation of the dye was performed with pulsed light of a mode-locked femtosecond Ti:sapphire laser (Mira 900 F, Coherent, Santa Clara, California) at a wavelength of 740 nm. The beam was focused on the sample with an oil-immersion microscope objective (PL APO 100×/1.4–0.7, Leica, Wetzlar). The built-in iris diaphragm allowed us to vary the NA between 0.7 and 1.4. The sample was mounted on a piezo-driven \( x, y, z \)-positioner (F-603 Light Line, Physik Instrumente, Waldbronn). Operating in a closed-loop mode, it allowed for positioning of the sample with 10-nm accuracy. The condition for a confocal arrangement was met by projecting an enlarged image of the focal region on a pinhole. A coloured filter glass (BG 39, Schott, Mainz) blocked the exciting light. The signal intensity behind the pinhole was measured by a photomultiplier tube.

Results

Axial responses were measured with numerical apertures of 0·7, 1·0, 1·2 and 1·4 (oil immersion). The responses are displayed as a function of the nominal focus position for glycerol in Fig. 1 and for water in Fig. 2. The left edge appears when the focus moves from the cover glass into the fluorescent sample. The right edge appears when the focus moves out of the sample.

The set of axial responses taken with Coumarin 138 in glycerol is shown in Fig. 1. The most prominent feature is
the intensity decline inside the sample for the data taken with NA = 1.4 (Fig. 1d). After passing through a thickness of about 9 μm of the sample, the signal intensity has dropped by 25% of its maximum intensity. This effect is also present in the responses with lower numerical apertures. For NA = 1.2 (Fig. 1c), the intensity is decreased by 7%, and for NA = 1.0 (Fig. 1b) the intensity decreased by 3%. For NA = 0.7 (Fig. 1a), no decline can be observed.

Figure 2 displays the set of axial responses for Coumarin 138 in water. For the highest aperture NA = 1.4 (Fig. 2d), a 25% drop in the maximum intensity occurred at a nominal focus position about 3 μm below the refractive interface. In contrast to glycerol, there are only minor changes when the numerical aperture is reduced from 1.4 to 1.2 (Fig. 2c). For NA = 1.0 (Fig. 2b), and NA = 0.7 (Fig. 2a), no decline can be observed.

**Fig. 1.** Glycerol responses of a two-photon confocal fluorescence microscope for numerical apertures of (a) 0.7, (b) 1.0, (c) 1.2 and (d) 1.4. The sample consists of Coumarin 138 immersed in glycerol (n2 = 1.47). The signal intensity in counts per channel is displayed as a function of the nominal focus position on the optical axis. The z-axis origin is arbitrarily set by the stage controller without reference to the sample geometry. The left edge shows the transition from the cover glass into the sample, the right edge from the sample into the microscope slide.

**Fig. 2.** Water responses of a two-photon confocal fluorescence microscope for numerical apertures of (a) 0.7, (b) 1.0, (c) 1.2 and (d) 1.4. The sample consists of Coumarin 138 immersed in water (n2 = 1.33). For explanations refer to Fig. 1.
In both sets of responses, the slope of the edges is larger for higher apertures. Since a part of the light is obstructed by the objective iris diaphragm, the responses have a lower maximum signal level for lower NA. This leads to the deterioration in the signal-to-noise ratio of the data for $NA = 0.7$.

The responses have been calculated theoretically for the parameters of the experiments. The results of three parameter sets for refractive index mismatch and numerical aperture are presented in Fig. 3. The data points are overlaid on normalized experimental data. For the glycerol responses with $NA = 1.2$ (Fig. 3a) and $NA = 1.4$ (Fig. 3b), the theoretical data follow the experimentally observed intensity decline. For water and $NA = 1.4$ (Fig. 3c), the calculations predict an intensity decrease of 45% at a nominal focus position 3 $\mu$m below the sample surface.

**Discussion**

The experimental and theoretical results show the strong effect of a refractive index mismatch in two-photon confocal fluorescence microscopy using high-NA objectives. Even for the moderate refractive index mismatch between glycerol ($n = 1.47$) and immersion oil ($n = 1.518$), the intensity decreased noticeably at depths exceeding 10 $\mu$m. For water ($n = 1.33$), which has a larger refractive index mismatch, the same intensity drop occurred at a depth of only 3 $\mu$m. Hence, imaging of aqueous specimens only a few micrometres thick will be affected in quality if performed with high-NA oil-immersion objectives.

The intensity decline is affected strongly by the numerical aperture. However, the mean pathlength of the light inside the sample is about the same for all apertures. This shows that absorption in the sample cannot be the cause of the effect. This is also supported by the fact that the calculations are able to predict comparable effects taking only the influence of a refractive index step into account.

By reducing the numerical aperture down to 1.0, the intensity decline is suppressed. The remaining variations in the image brightness may be tolerable for applications with a solely qualitative purpose. However, for thicker specimens further reduction in the numerical aperture might be necessary. For our layers less than 10 $\mu$m thick, a reduction to apertures below 1.0 provides no significant improvement (Figs. 1a and 2a). The broad edges indicate an overall poorer resolution.

The similarity in the Coumarin : water responses taken with NAs of 1.2 and 1.4, respectively, can be explained by the effect of total internal reflection at the coverglass–sample interface. This limits the effective aperture of an oil-immersion system to 1.3 when it is focusing into an aqueous specimen.

The theoretical model predicts the intensity decline inside the sample. For glycerol, the calculations are in quantitative accordance with the experiments. For water, the theory predicts a stronger decrease than the decrease found experimentally. Because Eq. (5) described the intensity decline for single-photon confocal fluorescence microscopy very well (Hell et al., 1993), we might speculate whether the simple model of squaring the excitation point spread function for two-photon excitation and multiplying by a similarly calculated detection point spread function in Eqs. (2) and (3) fully describes the practical situation. A variety of photophysical and photochemical processes not accounted for in the model might also play a significant role in two-photon excitation image generation. It is interesting to note that the effect of aberrations in two-photon excitation non-confocal imaging is similar to that in single-photon confocal imaging, both experimentally and theoretically.

**Conclusions**

It has been shown that the effects of refractive index mismatch are important in two-photon confocal
fluorescence microscopy. The observed and calculated intensity decline inside a specimen limits the use of high-NA objectives. The experimental results can be satisfactorily described by the theory.

For two-photon confocal fluorescence microscopy the following procedure is recommended: to obtain proper information about the fluorophore distribution in the whole specimen, the numerical aperture must not exceed 1.0. To resolve details that are located ≤ 10 μm below the cover glass, the aperture can be increased. A possible approach is to take the effects of index mismatch into account by subsequent image processing. To resolve details deep inside thick specimens, the immersion system must be matched to the refractive index of the specimen. For the quantitative study of thick aqueous specimens, water-immersion lenses are required for two-photon confocal fluorescence microscopy.

References