Effect of the specimen refractive index on the imaging of a confocal fluorescence microscope employing high aperture oil immersion lenses

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Submitted 19 October 1994, accepted 11 January 1995

Abstract. The effect of the specimen refractive index on the resolution, image brightness and axial scaling in confocal fluorescence microscopy is theoretically investigated for ten of the most popular fluorophores. The calculations are based on a vectorial theory based on Fermat’s principle. We show that the axial scaling of the image does not depend on the fluorophore used. Axial scaling factors are derived for objects embedded in glycerol and water. For each fluorophore, the maximum intensity and the full-width-half-maxima of the confocal point spread function are given as a function of the focusing depth. The full-width-half-maxima allow for an assessment of the axial and lateral resolution when deeper regions of the specimen are to be investigated.

Keywords: Confocal microscopy, fluorescence microscopy, refractive index mismatch, point spread function, resolution, image brightness, focal shift, axial scaling, aberration

1. Introduction

Confocal fluorescence microscopy is a versatile technique enabling three-dimensional imaging of thick transparent specimens. Three-dimensional imaging is achieved by applying a point-like light source for illumination and focusing the fluorescence light on a point-like detector. As a result of this arrangement, the point-spread-function of a confocal microscope $h_{\text{conf}}$ is given by the product of the excitation and detection point-spread function:

$$h_{\text{conf}} = h_{\text{ill}} h_{\text{det}}$$  \hspace{1cm} (1)$$

where $h_{\text{ill}}$ describes the intensity in the focal region and $h_{\text{det}}$ the image of the point-detector in the focus. The three-dimensional extent of $h_{\text{conf}}$ defines the resolution of a confocal fluorescence microscope. The extent of $h_{\text{conf}}$ depends mainly on the wavelength and the numerical aperture of the objective lens. To obtain the highest possible resolution, objectives with large numerical apertures are preferred. The highest apertures are achieved with oil immersion lenses.

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The refractive index of immersion oil and cover glass is about 1.52. Biological specimens, however, feature refractive indices ranging from 1.33 to 1.47. The pronounced difference in refractive index affects the image quality and resolution. The axial resolution and the image brightness decrease with increasing depth when high numerical apertures are applied (Rigaut et al 1990, Shaw and Rawlins 1991, Carlsson 1991). Furthermore, the actual position of the focus is not identical with its nominal value so that the image is artificially scaled along the optical axis (Carlsson 1991, Visser et al 1992).

For large numerical apertures, geometrical optics fails to describe refractive index mismatch induced effects. At low numerical apertures the effects are less pronounced, apart from the axial scaling of the image. Quantitative high aperture confocal fluorescence microscopy requires the refractive index mismatch to be taken into account. In a recent publication, the effects were consistently described as a consequence of the confocal arrangement (Hell et al 1993). Measurements and numerical calculations were performed showing good agreement of theoretical predictions with experiments. However, the investigations
Figure 1. Immersion objective lens focusing into a sample having a different index of refraction. If the refractive index of the sample \((n_2)\) equals that of the immersion system \((n_1)\), the focus is located at the nominal focus position (NFP). Due to refraction at the interface between the glass and the sample the focus is shifted to a different position, referred to as the actual focus position (AFP). Geometric optics, however, is not able to make quantitative predictions of the phenomenon for high numerical apertures.

were restricted to a single dye, namely Rhodamine B. Furthermore, the dependence on the excitation and fluorescence wavelength was not treated. For the growing community of confocal microscopy users it is of interest to get a feel for the effects for the most prominent dyes in the visible range. In this paper, we extend the investigations on the refractive index mismatch effects on ten different combinations of excitation and detection wavelength as they are encountered when using the most popular fluorophores in confocal fluorescence microscopy.

2. Theory

Figure 1 illustrates the situation commonly met in confocal fluorescence microscopy. The immersion system, consisting of objective, immersion oil, and cover glass, defines the nominal focus position. The nominal focus position (NFP) is the position at which one would expect the maximum of the confocal PSF \(h_{ill}\) in the absence of a refractive index mismatch.

If the refractive index of the specimen differs from that of the immersion oil, the interface between cover glass and sample forms a refractive index step. At the interface, the light travelling from the lens to the focus will be partly reflected and partly refracted thus apodizing the focused wave front. Furthermore, the mismatched medium affects the phase of the light causing deviations of the wave front from its initial spherical shape. The same applies to the wave fronts of the detected light. Thus, the confocal point spread function is altered. For example, the main maximum of \(h_{conf}\) is reduced and shifted to a new position referred to as the actual focus position (AFP).

The theoretical approach used in this work takes the vectorial character of light into account and holds for all numerical apertures. The refraction at the interface is described using Fermat’s principle of least optical path and Fresnel’s equations. The approach provides an equation for the confocal point spread function in the medium with different index of refraction (Hell et al 1993):

\[
h_{ill}(x, y, z) = \text{const} \left| \int_0^{2\pi} \int_0^\pi \sqrt{\cos \theta_1 \sin \theta_1} \times R \times \exp(ik_1s_1 + ik_2s_2) d\theta_1 d\phi \right|^2
\]

with

\[
R = \begin{bmatrix}
\tau_p \cos(\phi - \phi_0) \cos \theta_2 \cos \phi + \tau_s \sin(\phi - \phi_0) \sin \phi \\
\tau_p \cos(\phi - \phi_0) \cos \theta_2 \sin \phi - \tau_s \sin(\phi - \phi_0) \cos \phi
\end{bmatrix}
\]

(2)

and

\[
\tau_s = \frac{2 \sin \theta_2 \cos \theta_1}{\sin(\theta_1 + \theta_2)} \quad \tau_p = \frac{2 \sin \theta_2 \cos \theta_1}{\sin(\theta_1 + \theta_2) \cos(\theta_1 - \theta_2)}
\]

where \(\tau_s\) and \(\tau_p\) describe the change of the transmitted s- and p-polarized amplitudes according to Fresnel’s equations (Born and Wolf 1980). \(\theta_1\) is the angle of incidence, and \(\theta_2\) the refracted angle. The integration is performed over the objective aperture. \(\alpha\) denotes the semi-angle of the aperture, and \(\phi\) the azimuth angle. \(\phi_0\) describes the polarization of the illuminating plane waves. \(\phi_0 = \pi/4\) corresponds to circularly polarized or depolarized light. The dependence on the wavelength is taken into account by \(k_1 = 2\pi n_1/\lambda\) and \(k_2 = 2\pi n_2/\lambda\). \(s_1\) and \(s_2\) are the light paths in the first and in the second medium, respectively, and are calculated numerically according to Fermat’s principle of minimized total optical path length.

3. Parameters: fluorophores, mountants, numerical aperture

3.1. Fluorophores

The calculations were performed for the typical excitation and fluorescence wavelengths of ten commonly used dyes. The dyes are listed in table 1 together with the excitation and detection wavelengths used in the calculations. For excitation, the wavelengths of the popular Ar\(^+\)(Kr\(^+\))-lasers were chosen. The wavelength of the fluorescence maximum was taken for detection, with two exceptions, namely TRITC and CY5. These two dyes exhibit a small Stokes shift, so that the fluorescence is detected at wavelengths larger than the emission maximum. In this case, we have taken the transmission of the wavelength filters into account.

3.2. Mountants and numerical aperture

Glycerol and water were chosen as mounting media. Glycerol with a refractive index of 1.47 was chosen because it is a frequently used mountant. Water with a refractive index of \(n = 1.33\) was chosen because many biological
Refractive index mismatch effects for different fluorophores

<table>
<thead>
<tr>
<th>fluorophore</th>
<th>absorption maximum [nm]¶</th>
<th>emission maximum [nm]¶</th>
<th>excitation wavelength [nm]¶</th>
<th>detection wavelength [nm]¶</th>
</tr>
</thead>
<tbody>
<tr>
<td>coumarin 138</td>
<td>365</td>
<td>447</td>
<td>364 (Ar⁺)</td>
<td>447</td>
</tr>
<tr>
<td>DAPI</td>
<td>372</td>
<td>456</td>
<td>364 (Ar⁺)</td>
<td>456</td>
</tr>
<tr>
<td>FITC</td>
<td>490</td>
<td>520</td>
<td>488 (Ar⁺)</td>
<td>520</td>
</tr>
<tr>
<td>rhodamine B</td>
<td>545</td>
<td>565</td>
<td>514 (Ar⁺)</td>
<td>565</td>
</tr>
<tr>
<td>phycoerythrin</td>
<td>545</td>
<td>576</td>
<td>514 (Ar⁺)</td>
<td>576</td>
</tr>
<tr>
<td>acridine orange</td>
<td>490</td>
<td>590</td>
<td>488 (Ar⁺)</td>
<td>590</td>
</tr>
<tr>
<td>propidium iodide</td>
<td>530</td>
<td>615</td>
<td>514 (Ar⁺)</td>
<td>615</td>
</tr>
<tr>
<td>TRITC</td>
<td>541</td>
<td>572</td>
<td>568 (Kr⁺)</td>
<td>590</td>
</tr>
<tr>
<td>Texas red</td>
<td>596</td>
<td>620</td>
<td>568 (Kr⁺)</td>
<td>620</td>
</tr>
<tr>
<td>CY5</td>
<td>630–650</td>
<td>670</td>
<td>647 (Kr⁺)</td>
<td>690</td>
</tr>
</tbody>
</table>

3.3. Numerical methods and evaluation criteria

The integration across the aperture was carried out using a Gauss–Legendre algorithm. The optical path through the refractive interface was minimised by means of Newton’s algorithm. The calculations were performed on a 486 personal computer. The position of the principal maximum defines the actual focus position. The full width at half maximum (FWHM) of the principal maximum is chosen as a criterion for resolution.

4. Results

4.1. Survey of aberration effects induced by refractive index mismatch

The typical behaviour of the confocal point-spread-function $h_{conf}$ with increasing focusing depth (NFP) is exemplified in a series of confocal point spread functions calculated for FITC (figure 2). In the case of perfectly matched refractive indices, the intensity is concentrated in the main maximum. The relative height of the secondary maxima is 0.3%. The intensity maximum is located at the nominal focus position. The point spread function is symmetrical to the focal plane.

When focusing into glycerol or water, the refractive index mismatch affects the point spread function in several ways. The main maximum is shifted from its nominal position towards the objective lens. The height of the main maximum is strongly reduced (note the intensity in figure 2), and the height of the secondary maxima relative to the main maximum is increased compared to the unaberrated point spread function. For a point spread function of a nominal depth of 35 μm in glycerol, the main maximum reaches 18% of the unaberrated maximum height. The relative height of the first and second secondary axial maxima are 0.15 and 0.02, respectively. In general, the unaberrated main maximum as well as the point spread function is not symmetrical with respect to the focal plane. For the point spread functions at a NFP = 25 μm and NFP = 35 μm in glycerol, the axial width of the main maximum is increased. For a NFP of 45 μm, the width is decreased, while the relative strength of the secondary axial maxima increases to 16% and 4% for the first and second secondary axial maximum, respectively.

Figure 3 displays the decrease of the height of the main maximum, the broadening, and the axial shift of the main maximum with increasing focusing depth (NFP). The values for the points at $z = 0$ are obtained from the unaberrated point spread function. The decrease of the height of the main maximum means actually a decrease of brightness in the image. For glycerol, the calculations predict a loss of intensity less than 10% as long as the NFP is less than 10 μm. For an NFP of 30 μm the signal is reduced by 75%. At an NFP of 100 μm, the axial FWHM of the point spread function (figure 3(a)) is larger by a factor 2.4 and 4.2 for glycerol and water, respectively. The lateral FWHM in 100 μm depth (NFP) is increased by 30% in glycerol and by 70% in water. It is found for glycerol that at certain NFP (in figure 3(b) between 30 μm and 40 μm) the FWHM decreases slightly with increasing NFP.

Figure 3(c) shows that the actual focus position (AFP) is smaller than the nominal focus position (NFP) leading to an overall contraction of the axial image. The diagonal line represents the fully matched case where no refractive index induced aberrations are present.

4.2. Maximum intensity of the PSF

Figure 4 shows the dependence of the maximum intensity of the PSF on the NFP for the different fluorophores. One observes a general decrease of intensity for the first 30 μm and a shallow tail at a low level of intensity for an NFP larger than 50 μm for all fluorophores. The normalized
peak intensity in 10 μm NFP in water is 0.13, 0.18 and 0.23 for DAPI, Acridine Orange, and CY5, respectively.

4.3. Axial and lateral extent of the main maximum

Figure 5 displays the axial FWHM with increasing nominal focus position for ten different dyes. Each plot displays the results for glycerol and water. For example, the axial FWHM at a NFP of 40 μm in water is about 1 μm for DAPI, 1.2 μm for acridine orange, and 1.4 μm for CY5. In water, a monotonous increase of the axial FWHM up to 100 μm is found for all dyes. The axial FWHM increase is strongest at low NFP. For glycerol, the overall increase of the FWHM features slight oscillations with increasing depth. At the
first 20 μm focusing depth, the axial FWHM in glycerol is nearly constant.

The lateral FWHM in water increases monotonously for most of the dyes, as shown in figure 6. The UV-dyes coumarin 138 and DAPI are exceptions which exhibit a slight decrease of the lateral FWHM from 75 μm to 100 μm NFP. The lateral FWHM in glycerol shows slight oscillations resembling the oscillations of the axial FWHM. The axial and lateral oscillations are synchronous in terms of NFP.

4.4. Focal shift and axial scaling

Figure 3(c) shows that the actual focus position (AFP) is almost a linear function of the NFP. For a wavelength of 500 nm, the slopes are 0.958 in glycerol and 0.856 in water. We calculated the focal shift and the axial scaling factors for the ten selected dyes. This enabled the study of the axial scaling factor for changing excitation and detection wavelengths. We found that the change of the slopes z with the mean wavelength is Δs/d = -0.00001 nm^{-1} s Δλ for glycerol, and Δs/w = -0.00002 nm^{-1} s Δλ for water.

5. Discussion

We have calculated the effects of the refractive index mismatch for various fluorophores using a vectorial theory. The calculations gave us detailed values about the lateral and axial resolution, the decline in intensity and the axial scaling of the image for lenses fulfilling the sine condition.

5.1. Image brightness and resolution

The changes of the main maximum of the confocal pointspread-function, h_{conf}, with increasing NFP is qualitatively similar for all dyes. The FWHM varies, of course, with the excitation and fluorescence wavelength. Despite the similarity of the functions for various dyes, we could not establish an analytic function describing the FWHM of h_{conf} with a wavelength dependent scaling factor. This is due to the fact that the changes of h_{conf} are rather complex. The reason is that the point-spread-function h_{conf} is located only a few wavelengths from the interface. In this region, the laws of geometrical optics are not valid and simple scaling factors cannot be easily found or applied.

The comparison of the point spread functions in glycerol of figure 2 show that the FWHM is not a reliable criterion for defining the resolution in the presence of strong aberrations. The changes of the secondary maxima is not accounted for in this case. For example, the main maximum in glycerol changes with increasing NFP oscillating between a sharp and blurred appearance. These oscillations are well quantified by the FWHM. However, the relative height of the secondary maxima is still increasing while the FWHM decreases. Consequently, the decrease of the FWHM in glycerol does not automatically correspond to a resolution increase.

The FWHM oscillations in glycerol and their absence in water can be explained intuitively. The aberration effects are most pronounced for large numerical apertures and can be strongly reduced by reducing the numerical aperture. Therefore, the outer region of the focused light cone is most sensitive to refractive index changes. The aberrations observed in the focus are mostly dominated by the path length s_1 and s_2 determining the relative phase over the aperture. For glycerol, the moderate refractive index mismatch causes modifications of the phase that vary slowly across the aperture. Therefore, large regions of the light cone suffer a similar phase shift. This phase shift
Figure 4. Intensity maximum of the confocal point spread function as a function of the nominal focus position (NFP). The dots represent the relative height of the maxima for water ($n_2 = 1.33$), the open circles the results for glycerol ($n_2 = 1.47$) as mounting medium. An oil immersion objective lens with a numerical aperture of 1.3 was assumed. The dashed lines are cubic splines fitted to the calculated points.
Figure 5. Axial full width at half maximum (FWHM) of the confocal point spread function as a function of the nominal focus position (NFP). The dots represent the FWHM for water ($n_2 = 1.33$), the open circles the results for glycerol ($n_3 = 1.47$) as mounting medium. An oil immersion objective lens having a numerical aperture of 1.3 was assumed. The dashed lines are cubic splines fitted to the calculated points.
Figure 6. Lateral full width at half maximum (FWHM) of the confocal point spread function as a function of nominal focus position (NFP). The dots show the results for water ($n = 1.33$), the open circles the results for glycerol ($n = 1.47$) as mountant. An oil immersion objective lens with a numerical aperture 1.3 was assumed. The zero value of the vertical axis is suppressed in all plots.
changes slowly with increasing nominal focus position, giving rise to the pronounced oscillations in the FWHM. Following this argumentation, the period of the oscillations should be determined by the ratio of the optical path change and the wavelength. Indeed, this phenomenon is observed in figure 6: The larger the mean wavelength of illumination and detection, the longer is the period of the oscillations. For a watery medium, the large refractive index mismatch causes greater variations of the phase over the aperture angle thus averaging out possible oscillations of the FWHM.

The comparison of theory and experimental FWHM for high numerical apertures in former publications (Hell et al 1993, 1994a, b) showed that the experimental lateral resolution is in reasonable agreement with the theoretical limit, whereas the experimental axial resolution is systematically larger by approximately 40% than the theoretical prediction. This is probably due to the manufacturing tolerances of high aperture objective lenses, i.e. the lenses are specified with larger values (Juskaitis and Wilson 1994). The qualitative description of the phenomena is comprehensive and the other phenomena (axial scaling, intensity decline) in remarkable agreement with the experiment (Hell et al 1993). Therefore, we consider the results for the axial FWHM as useful and applicable when taking the deviation of the axial resolution into account.

5.2. Focal shift and axial scale

As the actual focus position is a linear function of the nominal focus position we were able to define an axial scaling factor both for glycerol and water. The variation of the axial scaling factor with the wavelength across the whole visible range is found to be weak. The axial scaling factor does not depend on the fluorophores used. A noticeable but probably practical unimportant deviation from the linearity of the axial scaling factor is found in the region closer than 5 μm to the interface. This is because the axial extent of the confocal point spread function is comparable to the focusing depth in that case.

6. Conclusion

We have described the lateral and axial resolution, the loss of intensity and the change of axial scaling of a confocal fluorescence microscope using oil immersion lenses for an index of refraction of the mounting medium different to that of the oil/cover glass system. Our investigations show that the wavelength of the light does not have a critical influence on the refractive mismatch induced axial scaling in confocal fluorescence microscopy. The axial scaling of the image is predicted not to depend on the type of fluorophore used. The axial scaling factors are 0.958 and 0.856 for glycerol and water, respectively. By multiplying the nominal axial distances with the axial scaling factors, the correct axial distances are found. For aqueous samples thicker than 40 μm immersion objectives should be used. The use of an objective lens with smaller numerical aperture can be a compromise that improves the resolution in higher depths at the expense of the resolution in lower depths. The calculated FWHM of the main maximum of the refractive index mismatched confocal point spread functions give a good estimate of the change of resolution that is expected with a given fluorescent label when imaging a glycerol mounted or watery specimen with a oil immersion lens of high numerical aperture.

Acknowledgments

This work was supported by the Academy of Finland and the research unit of Wallac Oy (Turku). We acknowledge valuable discussions with our colleagues P E Hänninen and Professor E Soini. Further, we would like to thank Professor C Cremer (Heidelberg) for his persistent interest in this topic and for encouraging us to carry out the present investigations.

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