Aberrations in confocal fluorescence microscopy induced by mismatches in refractive index

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Summary

The effect of refractive-index mismatch, as encountered in the observation of biological specimens, on the image acquisition process in confocal fluorescence microscopy is investigated theoretically. The analysis takes the vectorial properties of light into account and is valid for high numerical apertures. Quantitative predictions on the decrease of resolution, intensity drop and shift of focus are given for practical situations. When observing with a numerical aperture of 1.3 (oil immersion) and an excitation wavelength of 514 nm the centre of the focus shifts $1.7 \,\mu\text{m}$ per $10\,\mu\text{m}$ of axial displacement in an aqueous medium, thus yielding an image that is scaled by a factor of 1.2 in the axial direction. Furthermore, it can be expected that for a fluorescent plane 20 μ m deep inside an aqueous medium the peak intensity is 40% less than for a plane which is $10 \,\mu \text{m}$ deep. In addition, the axial resolution is decreased by a factor of 1.4. The theory was experimentally verified for test samples with different refractive indices.

Introduction

Motivation

In conventional fluorescence microscopy utilizing high-NA oil-immersion lenses the sample is flat and observed close to the cover glass. The light exiting the microscope objective traverses the immersion oil and the cover glass. The aqueous medium in which the cell is embedded has a different refractive index from that of the cover glass. The light is therefore refracted at the interface between the cover glass and the medium. The net effect of refraction depends on the observation depth. As long as observation is confined to the volume close to the cover glass the optical system performs well.

The introduction of confocal fluorescence microscopes has changed this standard situation as researchers can work with thick specimens. The need to look at flat specimens, simply because they are the only ones that can be observed well with a conventional fluorescence light microscope, has disappeared. Being able to record sections of reasonable quality inside the specimen moves the observation plane away from the region close to the cover slip and the light traverses a medium for which the optical system has not been designed. The light distribution in the observation volume is affected by the relative change in the refractive index and by the distance of the plane of observation from the cover glass. Since the light distribution close to the cover glass medium transition is considered perfect, any deviation results in a loss of intensity, resolution and a shift of the focus.

The fact that the resolution and the image brightness decrease under certain circumstances when the plane of observation is moved into deeper regions of the sample was noted by us very early on (Stelzer & Wijnaendts van Resandt, 1985; Hell & Wijnaendts van Resandt, 1989), and reported explicitly by others in a number of papers (e.g. Rigaut et al., 1990). Several interpretations of the confocal fluorescence image can be given. (i) The observed threedimensional (3-D) fluorophore distribution (this is a 3-D image) reflects the true distribution of the target. (ii) The fluorophore or the antibodies must diffuse throughout the sample. Diffusion barriers and insufficient labelling procedures cause a variation in the fluorophore distribution. The areas close to the medium receive more fluorophore than those deeper inside the cells. A gradient in intensity is therefore reasonable. (iii) Losses due to the scattering and the absorption of light in cells are not taken into account in the theories of image formation in a microscope. Since the cells have a structure they are heterogeneous and scatter light. Fluorophores are often found in very high concentra-



Fig. 1. The basic optical layout used for the calculations. The variables are explained in the text.

tions. The net effect is an intensity gradient along the optical axis. (iv) The refractive-index mismatch causes a spread of the confocal point spread function (PSF) and the photomultiplier behind a pinhole will detect a smaller fraction of the light than under ideal circumstances.

The capability of recording sections in a thick sample is usually exploited to record a series of sections and to use these for a 3-D reconstruction of some protein, DNA, lipid or fluid phase marker distribution. Since the fluorophore emission is proportional to the target concentration, 3-D images are also used to quantify the object. This raises a number of questions. How does the intensity depend on the distance of the plane of observation to the cover glass? How is the axial scale affected by the sample thickness? Are there methods to measure these effects and to overcome them by taking them into account during sample preparation, data recording and image data processing?

The paper will outline how the confocal PSF can be calculated for microscope objectives with a high NA in a second medium. This method is applied to calculate the PSFs for various media. Test objects were observed in a confocal fluorescence microscope and the results of calculations and experiments are compared. Intuitive physical explanations for the observed effects are given. Finally, the consequences for biology are outlined.

Confocal fluorescence microscopy

In confocal fluorescence microscopy a point-like light source is imaged into a transparent specimen. The light coming from the specimen is focused onto a point detector which is placed symmetrically to the point source (Wilson & Sheppard, 1984). Thus the ideal confocal fluorescence microscope works with physically well-defined illumination and detection wavefronts.

The ideal microscope objective converts the illumination wavefront into a spherical wavefront which moves towards the focus (Fig. 1). The 3-D light intensity distribution in the focus is described by the PSF of the microscope objective. The PSF depends on the wavelength and the numerical aperture. For the illumination process the PSF is referred to as the illumination PSF (I-PSF) $h_{\rm ill}(x, y, z)$ of the confocal system. The Cartesian coordinates x, y, z have their origin at the geometrical focus. The I-PSF is proportional to the probability that an excitation photon encounters a fluorescent molecule at the coordinate (x, y, z) in the focal

region. Due to the symmetrical arrangement of a confocal microscope a similar PSF $h_{det}(x, y, z)$ can be attributed to the detection process. The detection PSF (D-PSF) quantifies the probability that a fluorescent photon emitted at (x,y,z) is able to propagate to the point detector (Hell *et al.*, 1992). Since both events are required to occur, imaging in confocal fluorescence microscopy is determined by the product of both PSFs:

$$h_{\text{conf}}(x, y, z) = h_{\text{ill}}(x, y, z) \cdot h_{\text{det}}(x, y, z).$$
(1)

Resolution is determined by the 3-D extent of the confocal fluorescence PSF (CF-PSF) $h_{conf}(x, y, z)$ and described by a full-width-half-maximum (FWHM). For an ideal microscope the I-PSF, D-PSF and CF-PSF are symmetric with respect to the focal plane. Moreover, they have their highest value in the geometrical focal point.

Figure 1 shows a standard situation encountered in confocal fluorescence microscopy. Provided that immersion liquids of correct refractive index are used, aberrations can be neglected. In most cases, however, specimens have a index refractive (n_2) which is different to that of the cover glass immersion liquid system (n_1) . The transition between the cover glass and the specimen is a dielectric interface at which the light will be partly reflected and partly refracted. The light passing the interface undergoes changes in phase and amplitude, resulting in an aberration of the wavefronts and an apodization, respectively. The focal plane symmetry of both PSFs is lost and their extent is broadened. Moreover, the maximum of the PSFs is shifted (Born & Wolf, 1980, pp. 459–490).

It is therefore necessary to distinguish two focus positions. The nominal focus position (NFP) is the distance between the dielectric interface and the focus position under unaberrated circumstances. The change in NFP is therefore equivalent to a movement of the object relative to the objective lens, which is the quantity usually referred to when focusing into the object. The actual focus position (AFP), on the other hand, is the distance between the dielectric interface and the focus in the presence of aberrations (see Fig. 1). Hence, the focal shift is the difference between the NFP and the AFP. The focal shift changes the axial scale of the image. This should not, however, be confused with the focal shift occurring at low Fresnel numbers, as described by Li & Wolf (1981).

Theory

Previous contributions

During the last few years several workers have discussed the influence of the refractive-index mismatch on the confocal image acquisition process. Experimental investigations performed by Shaw & Rawlins (1991) and Carlsson (1991) demonstrated the importance of this issue. The focal shift due to the refractive-index mismatch has been reported by Carlsson (1991) and Visser *et al.* (1992). In both papers a geometrical approach was used to quantify the focal shift. This is based on the assumption that the position of the focus in the second medium is determined by the marginal rays of the aperture. This model predicts that the NFP is given by

$$NFP = \frac{\tan \alpha_1}{\tan \alpha_2} AFP = \frac{\tan [\sin^{-1}(NA/n_1)]}{\tan [\sin^{-1}(NA/n_2)]} AFP, \quad (2)$$

where α_1 is the aperture angle and therefore the angle of incidence of the marginal rays and α_2 is the corresponding refracted angle. n_1 and n_2 are the indices of refraction in the first and the second medium, respectively. NA is the numerical aperture of the microscope objective. For low numerical apertures Eq. (2) simplifies to

NFP =
$$\frac{n_2}{n_1}$$
 AFP. (3)

Carlsson (1991) reported that for an NA = 1.0 oil objective focusing into a water layer, Eq. (2) fails by 19%, whereas the low aperture approximation of Eq. (3) seems to predict the focal shift well. However, Visser *et al.* (1992) report good agreement of the results obtained by Eq. (2) with experimental data gained with fluorescent microspheres placed 100 μ m below the cover glass in a watery medium. It is therefore of interest to investigate whether Eq. (2) can be confirmed by a vectorial wave-optical theory.

A theoretical evaluation of the PSF obtained when focusing through a dielectric interface was given by Ling & Lee (1984). They tackled the problem numerically by decomposing the incident wavefront into a plane wave spectrum and applying a saddle-point approximation to the integration over the different directions of the plane waves. This theory takes the vectorial properties of light into account. However, their work had no significant impact on the theory of confocal microscopy.

The effect of aberrations on confocal imaging has been dealt with by Sheppard (1988) and Wilson & Carlini (1989), who used a vectorial and a scalar theory, respectively. In both papers the authors investigated the effect on axial resolution due to aberrations (e.g. spherical, coma, astigmatism) by considering an arbitrarily chosen aberration function ϕ . Wilson & Carlini (1989) reported the observation of calculated aberrations in confocal reflection microscopy. Later, Sheppard & Cogswell (1991) also reported the calculated effects observed in confocal reflection microscopy and indicated possibilities to balance these aberrations.

According to Sheppard & Cogswell (1991), the aberration function ϕ_{layer} , which describes the process of focusing through a dielectric layer with a refractive index n_2 embedded in a medium with a different refractive index n_1

is given by

$$\phi_{\text{layer}} = t \, k(n_2 - n_1) \bigg[1 + \frac{2n_1}{n_2} s^2 + 2(n_2 + n_1) \frac{n_1^2}{n_2^3} s^4 + \dots \bigg],$$
(4)

where k is the wave number with $k = 2\pi/\lambda$ and λ is the wavelength. $s = sin(\theta_1/2)$ where θ_1 is the angle of incidence of a ray inside the light cone and t is the thickness of the layer. The term in s^2 represents the defocus introduced by the layer, whereas the term in s^4 represents primary spherical aberration. It is worth considering whether Eq. (4) can be used to derive the PSFs for varying NFP by setting the layer thickness equal to the NFP. The first problem of this approach is that the second boundary which defines a thickness of the layer is not present. Also, since the PSFs have an axial extent (between 0.8 and 2.0 μ m at high NA) it is not straightforward to establish a single thickness in a thorough theory. Furthermore, the light propagating to (or from) a point P = (x, y, z) to the pinhole has to obey Fermat's principle. According to this, each point P = (x, y, z) is connected with a specific path for the light to travel to the pinholes. Since the points where the light crosses the interface (point I in Fig. 1), and the angle of incidence on the interface varies for neighbouring points, each point in the focal region has its own aberration function. We conclude that it is impossible to establish an aberration function ϕ across the aperture which describes the aberrations due to the interface for each point. The determination and decomposition of an aberration function ϕ is an approximation when addressing the problem of focusing into an object. Furthermore, in our opinion, work on aberrations still lacks a thorough comparison of the calculations with experimental data in both confocal reflection and fluorescence microscopy.

Calculating the PSF in a optically mismatched object medium by using Fermat's principle

Let us consider the focusing process as depicted in Fig. 1: according to the Huygens-Fresnel construction each point on the spherical wavefront is a source of secondary spherical wavelets. The field at an arbitrary point P = (x, y, z) in the focal region is determined by interference of the wavelets (Born & Wolf, 1980, pp. 370-375). When focusing into a matched medium $(n_1 = n_2)$ this yields

$$h(x, y, z) = \text{const}\left|\iint_{F} A(F) \frac{1}{s} K(\chi) \exp{(iks)} dF\right|^{2}.$$
 (5)

A(F) denotes the wavefront amplitude over the surface F of the spherical wavefront in the exit pupil. F denotes the surface of the spherical wavefront and dF is the surface element. s is the distance between the origin of the wavelet Q and the coordinate P = (x, y, z). For a matched second medium QP is a straight line and χ is the angle between the normal at Q and the direction QP. $K(\chi)$ is the inclination factor defined as

$$K(\chi) = -\frac{1}{2\lambda}(1 + \cos \chi). \tag{6}$$

When an aplanatic objective obeying the sine condition is illuminated with a plane wavefront the amplitude A(F) is cylindrically symmetric along the optical axis and varies with $\sqrt{(\cos \theta)}$ across the aperture (Richards & Wolf, 1959). θ is the polar angle of the light cone where $0 \le \theta \le \alpha.\alpha$ denotes the semi-angle of the aperture (Fig. 1). Using polar coordinates we write

$$h(x, y, z) = \operatorname{const} \left| \int_{0}^{\alpha} \int_{0}^{2\pi} \sqrt{(\cos \theta)} \frac{1}{s} K(\chi) \exp(iks) \sin \theta d\theta d\phi \right|^{2}.$$
(7)

In Eq. (7) the distance s = QP is a function of the angles θ and ϕ and focal length f. Since s in the range of the focal length (several millimetres) and the significant part of the PSF is confined to a small region of several micrometres (or less than 1 μ m for high-NA objectives), for a given point P = (x, y, z) in the focal region the variations of s across the aperture are in the range of $1^0/_{00}$. This variation is minor with respect to those of $\exp(iks)$. Therefore, 1/s can be regarded as constant and neglected for a normalized PSF. The same argument applies to the inclination factor $K(\chi)$. Since χ is expected to vary between 0 and 0.001, Eq. (6) shows that $K(\chi)$ can also be omitted without noticeable effects. These simplifications hold for all numerical apertures. Therefore, Eq. (7) can be written as

$$h(x, y, z) = \text{const} \left| \int_{0}^{\alpha} \int_{0}^{2\pi} \sqrt{(\cos \theta) \exp(iks) \sin \theta d\theta d\phi} \right|^{2}.$$
 (8)

For a paraxial approximation s is expanded and the terms higher than second order are omitted (Born & Wolf, 1980, pp. 436–437). Paraxial approximations are valid for low numerical apertures. For an exact description of s, however, a relatively simple expression can be derived. It complies with the spherical shape of the wavefront in the exit pupil and is valid for high numerical apertures in the framework of a scalar theory. This theoretical approach was used by Hell (1990) and is shown to predict the resolution of high-NA confocal reflection microscopy well.

In order to take the vectorial properties of the light into account, the amplitude A(F) is replaced by the electric field E(F). Since the electric field is required to be perpendicular to its direction of propagation, the electric field in the exit pupil E(F) is tangential to the spherical wavefront in the exit pupil (Fig. 1). Therefore, the field E(F) consists of components E_x , E_y , and E_z pointing in the x-, y- and z-



Fig. 2. Schematic view of the vector decomposition of the electric field *E*. (a) In a plane located in front of the exit pupil and parallel to the focal plane the incoming field *E* decomposes to a p-polarized component E_p and s-polarized component E_s . Due to the focusing process the component E_p is bent by the polar angle θ giving rise to a field pointing in the axial direction. This is shown in (b) by displaying a section of the spherical wavefront along the optical axis (see Eq. 9).

directions, respectively. Vector decompositions of the field in the exit pupil have been published by Hopkins (1943), Richards &Wolf (1959), van der Voort & Brakenhoff (1990) and Hell (1990). The decomposition of E(F) is based on the assumption that the p-polarized part of E(F), namely E_p , changes its direction by the angle θ when exiting the microscope objective, thus generating a field E_z which is parallel to the optical axis (Fig. 2). The s-polarized field E_s , however, is turned around its own axis and remains unaffected by the focusing process. E_s and the remaining component $E_{px,y}$ of E_p generate the components E_x and E_y which are parallel to the focal plane. If the incoming electric field is linearly polarized and has a polarization angle of ϕ_0 with respect to the x-direction, a straightforward geometric decomposition of E_p and E_s yields:

$$E(F) = \begin{bmatrix} E_{x}(F) \\ E_{y}(F) \\ E_{z}(F) \end{bmatrix} = |E(F)|$$

$$\times \begin{bmatrix} \cos(\phi - \phi_{0})\cos\theta\cos\phi + \sin(\phi - \phi_{0})\sin\phi \\ \cos(\phi - \phi_{0})\cos\theta\sin\phi - \sin(\phi - \phi_{0})\cos\phi \\ \cos(\phi - \phi_{0})\sin\theta \end{bmatrix}. \quad (9)$$

Thus, the electric field in the focal region is found by integrating each of these components across the spherical wavefront. The PSF is given by

$$h(x, y, z) = \left| \begin{bmatrix} E_x(x, y, z) \\ E_y(x, y, z) \\ E_z(x, y, z) \end{bmatrix} \right|^2$$
$$= \operatorname{const} \left| \int_{0}^{\alpha} \int_{0}^{2\pi} \sqrt{(\cos \theta)} \sin \theta \begin{bmatrix} E_x(F) \\ E_y(F) \\ E_z(F) \end{bmatrix} \right|$$
$$\times \exp(iks) d\theta d\phi \right|^2.$$
(10)

For an object medium with $n_2 \neq n_1$ the electric field undergoes changes according to Fresnel's equations (Born & Wolf, 1980, pp. 40-41).

$$\tau_{\rm s} = \frac{2\sin\theta_2\cos\theta_1}{\sin\left(\theta_1 + \theta_2\right)}; \quad \tau_{\rm p} = \frac{2\sin\theta_2\cos\theta_1}{\sin(\theta_1 + \theta_2)\cos\left(\theta_1 - \theta_2\right)}.$$
(11)

 θ_1 is the angle of incidence and θ_2 is the refractive angle in the second medium. τ_s and τ_p are the transmission coefficients for s- and p-polarized light. The change of the electric field amplitude when passing the interface can be taken into account by multiplying the s- and p-polarized components $E_{\rm s}$ and $E_{\rm p}$ with the transmission coefficients $\tau_{\rm s}$ and $\tau_{\rm p}$. The change in phase is quantified by applying Fermat's principle. According to Fermat's principle, the light follows a stationary path from its origin on the reference sphere *Q* to the point *P* in the second medium. Due to the geometry of the problem this is the least optical path. The least optical path consists of two parts s_1 and s_2 that are travelled by the light in the first and the second medium, respectively. When the least path crosses the dielectric interface at the point I, s_1 and s_2 are given by the distances QI and IP, respectively (Fig.1). s_1 and s_2 are functions of $\theta_1, \phi, n_1, n_2, x, y$ and z. The change in direction of the electric field when passing the interface requires the polar



angle θ to be replaced by θ_2 in the vectorial term in Eq. (10). For the integration across the spherical wavefront, the angle of incidence θ_1 has to be taken. Thus, Eq. (10) can be altered to yield the field in the second medium :

$$h_{n_1,n_2}(\mathbf{x}, y, z) = \left| \begin{bmatrix} E_{\mathbf{x}}(\mathbf{x}, y, z) \\ E_{y}(\mathbf{x}, y, z) \\ E_{z}(\mathbf{x}, y, z) \end{bmatrix} \right|^{2} = \operatorname{const} \left| \int_{0}^{\infty} \int_{0}^{2\pi} \sqrt{(\cos \theta_{1}) \sin \theta_{1}} \right|^{2}$$
$$\times \left[\tau_{p} \cos (\phi - \phi_{0}) \cos \theta_{2} \cos \phi + \tau_{s} \sin (\phi - \phi_{0}) \sin \phi \right]$$
$$\tau_{p} \cos (\phi - \phi_{0}) \cos \theta_{2} \sin \phi - \tau_{s} \sin (\phi - \phi_{0}) \cos \phi \right]$$
$$\tau_{p} \cos (\phi - \phi_{0}) \sin \theta_{2}$$
$$\times \exp (ik_{1}s_{1} + ik_{2}s_{2}) d\theta_{1} d\phi \right|^{2}, \qquad (12)$$

with $k_1 = 2\pi n_1/\lambda$ and $k_2 = 2\pi n_2/\lambda$. The integration is performed across the surface F of the spherical wavefront. Equation (12) is valid for all numerical apertures. The vectorial term in Eq. (12) indicates that the PSF is generally not symmeric with respect to the optical axis. The size and the location of the PSF, however, will be determined foremost by the phase term exp $(ik_1s_1 + ik_2s_2)$. For a given situation all variables in Eq. (12) are known, except s_1 and s_2 . In contrast to the single medium case where a simple analytic expression for s is found, a numerical method must be used for calculating s_1 and s_2 . This can be accomplished by calculating the crossing point I by means of Newton's algorithm. Fig. 3. Two different views of the 3-D confocal point spread function for an NFP of $15 \,\mu\text{m}$ in water. The picture on the left-hand side is a contour plot with isophotes at 0.7, 0.5, 0.3, 0.1, 0.01 and 0.001. The picure on the right-hand side is a surface plot of the same data set. While the contour plot can be used to extract quantitative information, the locations of the minima and maxima are clearly visible in the surface plot. The focal shift is 2.6 μ m and the full width half maximum in the axial direction is 0.88 μ m, which is about twice as large as under ideal circumstances.

The I-PSF when observing a mismatched medium is given by the modulus squared of the field described in Eq. (12):

$$h_{\rm ill}(x, y, z) = h_{n_1 n_2}(x, y, z)$$

= $|E_x(x, y, z)|^2 + |E_y(x, y, z)|^2 + |E_z(x, y, z)|^2.$
(13)

For the calculation of the D-PSF, $h_{det}(x, y, z)$, the longer fluorescence wavelengths and the random polarization the fluorescence light has to be taken into account. If the fluorescence light is assumed to be monochromatic for simplicity, the D-PSF is readily calculated by using a polarization angle ϕ_0 of $\pi/4$ and applying a longer wavelength. The polarization angle ϕ_0 of $\pi/4$ complies with the random polarization of the fluorescence light, because it is shown to be equivalent to integrating over the polarization angle ϕ_0 (van der Voort & Brakenhoff, 1990). In addition, the D-PSF is calculated with a uniform amplitude across the spherical detection wavefront (Visser et al., 1991) in order to take into account the uniform spherical shape of the emitted fluorescence wavefront. This is equivalent to omitting the amplitude factor $\sqrt{\cos\theta}$ in Eq. (12).

Theoretical results

The following calculations were performed numerically for an oil-immersion microscope objective with an NA = 1.3using Eqs. (12) and (13). The excitation light was assumed



Fig. 4. Contour plots of confocal point spread functions (C-PSFs) and their respective z-responses for different NFPs in water. The contour plot for z = 0 assumes the ideal conditions of immersion oil. Each of the C-PSFs was normalized to peak intensity. The z-response curves plot the laterally integrated intensity as a function of z. These curves are normalized to the ideal condition encountered at z = 0. The contour plots show that the confocal observation volume is increased when the probing beam penetrates deeper parts of the object. The spread of the C-PSFs occurs mainly along the optical axis and not laterally. The parameters for the computations are derived from the following situation: rhodamine 6G is dissolved in water, which has a refractive index of 1·33. The fluorophore is excited with a wavelength of 514 nm and is observed at a wavelength of 590 nm. The microscope objective lens has a numerical aperture of 1·3 and its immersion oil has a refractive index of 1·518. The illumination and the detection pinhole are both infinitely small. The illumination and detection light are both randomly polarized. The sample has a single flat boundary in the cover glass orientated towards the microscope objective lens; it is otherwise infinitely large. The contour plots are drawn with an aspect ratio of Z/R = 25 nm/20 nm.

to be either circularly or randomly polarized ($\phi_0 = \pi/4$), since this is the case in the confocal microscopes at EMBL, Heidelberg. In this case both the I-PSF and the D-PSF are cylindrically symmetric with respect to the optical axis. The excitation wavelength was 514 nm, whereas the fluorescence emission was considered to occur at 590 nm. Figure 3 shows two different views of a section of the confocal fluorescence point spread function (CF-PSF) along the optical axis for an NFP of $15 \,\mu\text{m}$ in water $(n_2 = 1.33)$. The picture on the left-hand side is a contour plot showing lines of equal intensity (isophotes). On the right-hand side a surface plot of the same data set is shown. The data were normalized to unity. Note the asymmetry with respect to the lateral plane containing the maximum of the CF-PSF. Towards the cover glass pronounced axial lobes are found. The first axial lobe has a height of 0.12, the second a height of 0.05.

The upper diagram in Fig. 4 shows contour plots of CF-PSFs calculated for three different NFPs in water $(n_2 = 1.33)$: 5 μ m, 10 μ m and 20 μ m, for (b), (c) and (d), respectively. Figure 4(a) shows the unaberrated CF-PSF in a perfectly matched medium, where no aberrations are present. It is clear, that the axial extent increases dramatically with increasing NFP. The spread of the CF-PSF occurs mainly along the optical axis and not laterally. Since water has a lower refractive index than oil the focus is shifted towards the microscope objective. This is also displayed in Fig. 4: the CF-PSFs for (b) 5 μ m, (c) 10 μ m, and (d) 20 μ m NFP are displaced by (b) 1.0 μ m, (c) 1.83 μ m and (d) 3.3 μ m, respectively.

To quantify the decrease of axial resolution when observing lateral planes for a given NFP, the z-responses corresponding to the CF-PSFs of Fig. 4 were calculated. The confocal z-responses are defined as follows:

$$I^{\rm NFP}(z) = \iint h_{\rm ill}^{\rm NFP}(x, y, z) \cdot h_{\rm det}^{\rm NFP}(x, y, z) \, \mathrm{d}x \, \mathrm{d}y. \tag{14}$$

Again, Fig. 4(a) shows the unaberrated z-response when



focusing, for example, into immersion oil, whereas Fig. 4(b-d) show the corresponding aberrated z-responses as they are obtained for (b) $5 \mu m$, (c) $10 \mu m$ and (d) $20 \mu m$ NFP in water. The z-responses are normalized to the unaberrated z-response shown in Fig. 4(a). Note the decrease in peak intensity with increasing NFP. The peaks of the aberrated z-responses are reduced to (b) 0.6, (c) 0.39, and (d) 0.23. This means the signals from lateral fluorescent planes decrease the deeper they are observed in an optically mismatched medium. Furthermore, the z-responses are broadened with increasing NFP, leading to poorer axial resolutions when deeper regions of the object are examined. The FWHMs of the z-responses are $0.53 \mu m$ for the unaberrated (a), $0.68 \mu m$ for $5 \mu m$ NFP (b), $0.9 \mu m$ for $10 \mu m$ NFP (c), and $1.23 \mu m$ for $20 \mu m$ NFP (d).

Fig. 5(a) shows the calculated AFP versus the NFP for a fluorescent dye immersed in an oil $(n_2 = 1.518)$, glycerol $(n_2 = 1.47)$ and water $(n_2 = 1.33)$ medium when observing with an NA = 1.3 oil-immersion objective. Since glycerol and water have a lower refractive index than immersion oil the focus is shifted towards the cover glass, resulting in an AFP that is smaller than the NFP. For an NFP of 15 μ m in an aqueous medium the aberration yields a



Fig. 5. Changes in the confocal point spread function with increasing NFP. (a) The actual focus position (AFP) versus the nominal focus position (NFP) in oil, glycerol and water. This causes an increase in the apparent sample thickness. (b) The maximal intensity of the z-response, which is the maximal brightness of a thin layer, decreases in glycerol and in water. (c) The full width half maximum of the z-response increases in glycerol and in water. This means that the axial edge resolution of a confocal fluorescence microscope becomes worse. The axial (d) and the lateral (e) full width half maximum of the PSF in glycerol and in water for increasing NFP.

focal shift of $2.575 \,\mu\text{m}$ in the direction of the microscope objective, whereas for an NFP of $30 \,\mu\text{m}$ a focal shift of $4.725 \,\mu\text{m}$ is expected. Note the nearly linear relationship between the NFP and the AFP. This leads automatically to an elongation of the sample in the axial direction. Therefore, our theory predicts that the axial distances of a watery specimen are elongated by a factor of 1.2.

Figure 5(b-e) show the theoretically predicted change of the CF-PSF with increasing NFP when the specimen has a refractive index close to that of glycerol and water. They describe the decrease of intensity and the loss of resolution when penetrating deeper into the specimen. The theoretical results are also summarized in Table 1.

Experimental results

High numerical apertures

Three fluorescent seas were prepared by dissolving rhodamine 6G (Eastman Kodak Co., 1991) in immersion oil $(n_2 = 1.518)$, glycerol $(n_2 = 1.47)$ and water $(n_2 = 1.33)$. The respective thicknesses were of the order of $40 \,\mu$ m, $75 \,\mu$ m and $85 \,\mu$ m. The fluorescent seas were

NFP (µm)	Glycerol				Water					
	Focal shift (µm)	Intensity	FWHM z-response (µm)	FWHM PSF axial (µm)	FWHM PSF lateral (µm)	Focal shift (µm)	Intensity	FWHM 2-response (µm)	FWHM PSF axial (µm)	FWHM PSF lateral (µm)
0	0	100	0.53	0.475	0.16	0	100	0.53	0.475	0.16
5	-0.28	95	0.53	0.475	0.16	-1.0	60	0.68	0.58	0.2
10	-0.55	91	0.53	0.475	0.16	-1.83	39	0.9	0.7	0.2
15	0.83	78	0.555	0.475	0.16	-2.575	28.5	1.08	0.88	0.23
20	-1.1	62	0.65	0.5	0.18	-3.3	23	1.225	0.93	0.24
25	-1.33	50	0.81	0.575	0.18	-4.022	19	1.37	1	0.24
30	-1·5 4	40	0.97	0.775	0.5	-4.725	16.6	1.45	1.11	0.24
50	-2.3	31	1.0	0.725	0.2	-7.575	11	1.795	1.375	0.24

Table 1. Summary of the calculations performed for water and for glycerol.

The conditions for the calculations are described in the section on theoretical results. The reference position at an NFP of 0 μ m assumes ideal conditons encountered during the observation of rhodamine 6G immersed in immersion oil. The NFP is the distance from the dielectric interface to the focus position for aberration-free conditions. The focal shift is the difference between the NFP and the AFP. The intensity is the peak intensity collected with a point object. The full width half maximum (FWHM) of the central peak of the z-response indicates the resolution of an edge along the optical axis. The axial and the lateral full width half maximum of the point spread function indicates the point resolution.

mounted between cover glasses and microscope slides (Fig. 1). The axial sea response is gained experimentally by scanning the sea in the axial direction and displaying the data as a function of the stage displacement (NFP). Therefore, the sea response is a function of the NFP and can be calculated by integrating the contributions of each point in the sea for varying NFPs:

$$I_{\text{sea}}(\text{NFP}) = \iiint h_{\text{ill}}(\text{NFP}, x, y, z) \cdot h_{\text{det}}(\text{NFP}, x, y, z) \, dx \, dy \, dz.$$
(15)

Sea responses were recorded with the compact confocal microscope (CCM) at the EMBL, Heidelberg (Stelzer, 1990), using an NA = 1·3 oil ($n_2 = 1.518$) immersion objective (Zeiss Plan-Neofluar 100×). The fluorophore was excited with $\lambda_{exc} = 514$ nm. The mean fluorescence wavelength was in the range of 590 nm. The detection pinhole of the CCM was adjusted to a diameter of 30 μ m corresponding to 67% of the magnified Airy disc (Wilson & Carlini, 1988).

Figure 6 shows sea responses of rhodamine 6G immersed in: (a) oil, (b) glycerol and (c) water. The undulating lines follow the experimental data. The smooth lines show the curves calculated with Eq. (15). The slope on the left-hand side is caused by entering the sea of fluorophore, while the slope on the right-hand side is caused by exiting the fluorescent sea. The signal intensity decreases with increasing NFP for glycerol and water. In the glycerol sea the intensity is reduced by 30% for an NFP of 50 μ m, while in water the intensity drops by 61% for the same NFP of 50 μ m. The drop in intensity does not occur when the refractive index is matched to that of the cover glass. An example is shown in Fig. 6(a) where the dye was dissolved in immersion oil. In Fig. 7 the response of the cover glass/glycerol sea interface and of the glycerol sea/microscope slide interface in Fig. 6(b) are displayed on a larger scale. The poorer slope of the response to the glycerol sea/microscope slide interface indicates the decrease of axial resolution after an NFP of 70 μ m in glycerol (see Table 2).

The derivatives of the interface responses should not however be confused with the theoretical z-responses shown in Fig. 4. According to Eq. (14), z-responses are calculated for an infinitely thin fluorescence plane moving axially while keeping the NFP and therefore the CF-PSF constant, whereas sea responses (Eq. 15) are recorded by moving the whole sample, thus changing the NFP and therefore also the CF-PSF. Since it is very difficult to conceive an experiment where a fluorescent sample is moved axially while keeping the aberrating layer thickness constant, the measurement of the z-responses could not be carried out. The sea responses served as suitable and easyto-prepare objects for testing the reliability of the theoretical predictions. It is clear that, due to the dependency of the C-PSF on the NFP, the z-responses, and not the edges of the sea responses, determine the axial resolution for thin fluorescent planes in a mismatched medium.

Low numerical apertures

Figure 8 shows the sea response of the fluorescent water sea with a numerical aperture of 0.8 oil immersion. The



Fig. 6. Sea response of a confocal fluorescence microscope for rhodamine 6G immersed in (a) immersion oil $(n_2 = 1.518)$, (b) glycerol $(n_2 = 1.47)$ and (c) water $(n_2 = 1.33)$. The slope on the left-hand side shows the transition from the cover glass close to the microscope objective to the layer of fluorophore. The transition on the right-hand side is from the fluorophore solution to the microscope slide. The agreement between the experiments and the theoretical calculations is good.

undulating line shows the experimental data while the smooth line shows the calculated data. The calculated data



Fig. 7. The (a) upper and (b) lower boundary of a fluorescent gycerol sea as observed with a confocal fluorescence microscope. This detailed view has been calculated and observed under the same conditions as described above for glycerol. The sample thickness in terms of NFP is 74 μ m, which corresponds to an actual thickness of 70 μ m. The edge resolution decreases 2.5-fold and the intensity decreases by 23%. The agreement between theoretical and experimental results is good.

follows the slope and the intensity variations well. Figure 9 shows the sea response of a water sea recorded with two different numerical apertures. To perform this experiment a Zeiss Plan-Apochromat 100x oil objective with a variable aperture was chosen. Because of total internal reflection on the cover glass/water interface $(n_1 = 1.518/n_2 = 1.33)$ the numerical aperture of 1.4 is effectively 1.33. The images were normalized to have the same maximal height. According to the data shown in Fig. 9 the higher NA lens provides a higher axial resolution close to the cover glass but the drop in intensity becomes significant. The lower NA system suffers no decrease in intensity.

Focal shift and axial scaling of the image

For a direct proof of the focal shift as a function of the refractive-index mismatch two fluorescent seas were



Fig. 8. The sea response of the fluorescent water sea with a numerical aperture of 0.8 (oil immersion). The undulating line shows the experimental data while the smooth line shows the calculated data.



Fig. 9. The sea response of a fluorescent water sea recorded with two different numerical apertures: NA = 1.4 (effectively 1.3, because of total internal reflection) and NA = 0.8.

mounted in a single sample. A drop of rhodamine 6G dissolved in water and a drop of rhodamine 6G dissolved in immersion oil were mounted between a cover glass and a slide. The solutions did not mix and two xz-images covering the complete sample were recorded close to their clearly visible interface (Fig. 10). Using an NA = 1.3 oil objective, two experiments with two different sample thicknesses were

carried out. In the first experiment the boundary between the sea and the slide was located at [NFPs of 138 μ m and 166 μ m for oil and water, respectively. The NFP for oil can be regarded as the true value because the refractive indices are matched when using an oil-immersion objective. In the second experiment a thinner sample was used. The lower boundary of this sample was located at 60 μ m NFP for oil and 72 μ m NFP for water. Accordingly, the focal shifts were 28 μ m for the thicker sample and 12 μ m for its thinner counterpart. Therefore, the refractive-index mismatch scaled the first image by a factor of 1.203 and the second by 1.2, in accordance with theoretical predictions. Since the NFP can be established with an accuracy better than 1 μ m, a rather pessimistic estimate of the relative measurement error is 1%.

Discussion

Comparison of theoretical and experimental results

The theory presented in this paper describes the effects of aberrations occurring when focusing into an optically mismatched medium. It predicts the behaviour of the CF-PSFs in the mismatched medium: the loss of resolution, the



Fig. 10. x-z sections through seas of rhodamine 6G immersed in water (left) and immersion oil (right) mounted as one sample. The laser beam enters the sample through the cover glass (top) and exits into the slide (bottom). The lower edge for the water is located at an NFP of 72 μ m (left). For the oil layer the NFP is 60 μ m ($\lambda_{exec} = 514$ nm, $\lambda_{em} = c$. 590 nm). The lower edge for the water is brighter due to sedimentation of the fluorophore.

	Water (NA	= 0.8)	Water (NA	= 1.3)	Glycerol (NA = 1.3)	
	Theory	Experiment	Theory	Experiment	Theory	Experiment
Upper edge (μm)	1.2	1.5	0.6	1.0	0.6	1.0
Lower edge (μm)	1.5	2.0	3.1	5.7	1.6	2.3
Upper edge/lower edge	1.3	1.3	5.2	5.7	2.6	2.3

Table 2. Summary of the axial edge slope for the fluorescent water and glycerol seas.

The values represent the distance along the optical axis between the 25% and 75% thresholds of the upper and lower boundary of the fluorescent layer. The last row shows the ratios of the upper and lower boundary slopes.

focal shift, the scaling of the image and the drop in intensity. Figures 6–8 show a very good agreement between the experimental curves and the calculations, proving the reliability of Eq. (12). A good agreement is also found for the axial focal shift when focusing with an NA = 1.3 oil objective in a watery medium. The experiment revealed that 60-and 138 μ m-thick watery samples were elongated by a factor of 1.2 in the axial direction. Theory predicts a factor of 1.18 for samples of about 50 μ m thickness and 1.2 for samples of about 30 μ m thickness. In a good approximation the focal shift is proportional to the NFP, allowing for a linear correction of the image. The appropriate scaling factor correction is 1/1.2 = 0.83 for this case.

Slight deviations of the theoretical predictions from the experimentally gained data are found at the axial edges and in the deepest regions of the fluorescent water sample. A possible reason for the somewhat lower values of the experimental data in the deepest regions of the water sample (Fig. 6c) is the dependency of the aberrations induced by mismatches in refractive index on the wavelength. In our calculations a monochromatic fluorescence wavelength has been assumed. In practice the fluorescence emission has a bandwidth of more than 50 nm. The chromatic effects are expected to blur the D-PSF, vielding a lower intensity and a flatter lower axial edge. The ratios of the slopes of the upper and lower axial edges agree well, but the axial edges are somewhat less steep in the experiment than predicted by theory (see Fig. 7, Table 2). Another reason for this phenomenon might be that our theory does not consider effects occurring in the vicinity of the axial edge. Electrodynamics requires evanescent waves inside the second medium. The excitation light considered to be reflected at the dielectric interface penetrates the second medium by a depth in the order of $\lambda_{\rm exc}$. Thus, it excites fluorophore molecules and changes the fluorescence response.

An important result of these investigations is that the decline in intensity when focusing into deeper regions of the sea can be explained solely by aberrations. The absorption coefficients of the fluorescent glycerol and water seas used in these experiments were measured using a spectrometer. We calculated that less than 1% of the incoming light was absorbed when penetrating these samples. This is below the noise level of our experimental data. The negligible effect of absorption is also proved in the experiment shown in Fig. 9, where two different apertures were used with an identical sample. Since aberrations are stronger at higher numerical apertures, the intensity drop can be avoided by switching to a lower numerical aperture.

An intuitive explanation of aberrations induced by mismatches in refractive index

The aberration-induced decline in intensity with increasing NFP can be explained easily as follows. According to Eq. (1) in a confocal system the contribution of each point in the focal region is weighted by the product of the illumination and detection PSFs. Therefore, in contrast to a conventional epi-fluorescence microscope, in a confocal microscope the more remote and inherently weaker parts of the PSF are further reduced due to the multiplication. A major effect of the aberrations is a spreading of the I-PSF and D-PSF with respect to their aberration-free counterparts. The more remote parts of the I-PSF and D-PSF are therefore strengthened at the expense of the main maximum. The multiplication, however, discriminates these parts in the focal region causing a loss of detectable intensity.

If the confocal point detector is replaced by an infinitely large detector, the imaging is solely determined by the I-PSF and the decrease in intensity does not occur. Therefore, the decline in intensity should not be confused with the decrease of the main maximum of a PSF when aberrations are present. The latter also occurs in conventional microscopy, but does not decrease the total intensity. The image is merely blurred. The intensity loss is a result of the confocal arrangement.

Multiplication, however, has another consequence which is advantageous. Figure 11 shows axial sections of an aberrated D-PSF (Fig. 11c), describing conventional



Fig. 11. Axial sections of (a) an aberration-free confocal fluorescence point spread function (CF-PSF), (b) an aberrated CF-PSF and (c) an aberrated detection point spread function (D-PSF) describing imaging in conventional fluorescence microscopy. Note that in (a) and (b) the points more remote from the main maximum are suppressed. The asymmetry of the aberrated CF-PSF (b) is therefore not as evident as in its conventional counterpart (c).

epifluorescence imaging, and corresponding unaberrated (Fig. 11a) and aberrated (Fig. 11b) CF-PSFs. The aberrated CF-PSF in Fig. 11(b) is calculated for the same aberrations as the conventional PSF in Fig. 11(c). The unaberrated CF-PSF (Fig. 11a) is symmetric with respect to the focal plane. This is not the case in Fig. 11(c) where strong axial lobes are present. It is interesting to note that the aberrated CF-PSF is also fairly symmetric. This phenomenon was demonstrated experimentally by Shaw & Rawlins (1991) who investigated the PSF using fluorescent beads. An explanation for this is given when looking at the remote parts of the PSFs. In contrast to its conventional counterpart, the confocal system discriminates these regions. Therefore, the asymmetry is less evident in the CF-PSF.

As a result, in a confocal fluorescence system the effect of aberrations on the resolution is not as important as in a conventional system. This advantage, however, is gained at the expense of signal intensity when high numerical apertures are employed. When deeper regions of the object are observed with high numerical apertures, increasing the detection pinhole increases not only the signal but also the influence of aberrations on the resolution. The increasing influence of the aberrations with increasing detector pinhole size is in accordance with the conclusions drawn by Wilson & Carlini (1989) for the axial response in confocal reflection microscopy.

Our theoretically determined and experimentally verified

scaling factor of 1.2 is closer to the geometric small aperture factor of 1.14 in Eq. (3) than to the factor of 2.8 suggested by the marginal ray model (Eq. 2; Visser et al., 1992). We therefore believe that the marginal rays play a rather minor role in determining the aberrated focus positions. Having a high angle of incidence, the marginal rays can be expected to enlarge the axial width of the aberrated CF-PSF leading to a much poorer axial resolution (see Table 1). This view is also supported by the experiment shown in Fig. 9 where the same water sample was recorded with an NA of 1.4 (1.33 effective) and 0.8 (oil immersion). The bottom edge for the NA = 1.4 recording is shallower than in its NA = 0.8counterpart. This can be attributed to the presence of axial side lobes as shown in Fig. 4. The apparent thickness of the water sample (the NFP of the bottom layer), however, is barely affected and a significant change in the axial scale does not occur. This is in contrast to the predictions by Visser et al. (1992) (Eq. 2) where a change of the numerical aperture should severely alter the axial scaling. In addition, an infinitely high scaling factor is predicted for the NA = 1.4recording in this special case. Such a scaling is clearly not present. Our theory is supported by the experiments described by Carlsson (1991) who found that the focal shift of an NA = 1.0 oil objective focusing into water is much less than predicted by the marginal ray model, supporting his speculations on this subject.

It is also interesting to note that according to our theory

the axial scaling factor increases slightly with decreasing NFP (see focal shift in Table 1). This indicates that it is not possible to remove the axial scaling by observing close to the cover glass. Indeed, it can be expected that the regions close to the cover glass are slightly more scaled than those in deeper regions of the specimen.

The present work indicates that aberrations which are due to the mismatch of the specimen refractive index are always present and in many cases more important than absorption when objectives of high numerical aperture are utilized. For a quantitative analysis of 3-D images of thick specimens they have to be taken into account.

Conclusions

What are the consequences for the application of confocal fluorescence microscopy in cell biology? If the cells are kept in an aqueous medium with a refractive index of 1.33, observed with numerical aperture of 1.3 (oil) and an excitation wavelength around 500 nm, the axial edge resolution decreases by a factor of 1.4 and the intensity decreases at a rate of 40% between 10 μ m and 20 μ m NFP. The axial distances have to be scaled by a factor of 0.83. If we assume the beam moves through the cell and the refractive index is close to 1.47, the axial resolution decreases by a factor of 1.2 per 10 μ m, the intensity decreases at a rate of 30% per 10 μ m and the axial scaling factor is 0.96.

Whenever accurate 3-D measurements have to be made it is important to be aware of the axial scaling factor. In cellular objects, axial distances up to 20 μ m may be obtained easily. Assuming refractive indices similar to that of water, the refractive-index mismatch will cause focal shifts of up to 3 μ m. Axial scaling is important when models of 3-D all-type specific genome organizations are investigated experimentally. A concept proposed by Krystock & Puck (1990) localizes the active genes preferentially at the nuclear periphery and sequesters inactive genes internally. A verification requires reliable 3-D distance measurements between fluorescing targets within the chromosome domains. Since the typical domain diameter of an individual human chromosome is in the order of micrometres (Bischoff et al., 1993), a focal shift of 750 nm might lead to erroneous conclusions.

Image distortion and intensity losses cannot be avoided during the observation of biological specimens kept in an aqueous medium when observed with high-NA oil immersion lenses. For low numerical apertures aberrations have less effect on confocally recorded images. However, in this case the advantage of confocal fluorescence microscopy is less obvious. Water-immersion lenses should therefore be considered when the aqueous medium is penetrated more than $10 \,\mu$ m. The situation is then similar to the observation of fluorophores immersed in immersion oil which has perfect conditions. If the use of an oil-immersion lens cannot be avoided the problem of a decrease in intensity can be overcome by increasing the pinhole diameter. This, however, is gained at the expense of the resolution and an increase in the influence of the aberrations. It is therefore not generally recommended. The effects introduced by the optical mismatch, are, however, quantifiable on the basis of Eq. (12) and can be taken into account when processing and analysing image data sets.

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