Improvement of lateral resolution in far-field fluorescence light microscopy by using two-photon excitation with offset beams

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A method is described of increasing the resolution in far-field fluorescence light microscopy by a factor of 2. Calculations show that a lateral resolution of 75 nm is achieved with a lens of numerical aperture of 1.4 (oil immersion) by a two-photon excitation mode with each photon stemming from displaced focused light beams of λ = 500 nm and overlapping main maxima.

1. Introduction

Far-field light microscopy offers several advantages when compared to other microscopical techniques especially for the investigation of biological specimens [1]. Its major shortcoming, however, is its diffraction limited resolution which is considered to be in the range of 150–200 nm [2]. This has led to the development of near-field scanning optical microscopes which avoid diffraction by employing a scanning tip placed close to the specimen surface [3]. In addition to being limited to the investigation of the specimen surface, near-field scanning optical microscopes require a tight control of the scanning tip. Therefore resolution enhancement in far-field microscopy is of major interest. By introducing the 4Pi-confocal microscope [4–7] the present author has demonstrated an increase of the axial resolution in far-field light microscopy by a factor of 3–7. This latter concentrates on the increase of the lateral resolution. It shows that it is possible to significantly increase the lateral resolution in fluorescence microscopy without increasing the aperture or decreasing the wavelength.

2. Method

When the vectorial properties of light are taken into account the normalized intensity distribution of a linearly polarised plane wavefront focused to a point \((x=0, y=0, z=0)\) is given by [8]

\[
h(u, v, \varphi) = |(e_x, e_y, e_z)|^2 = |I_0(u, v) + I_2(u, v) \cos 2\varphi, I_2(u, v) \sin 2\varphi, -2iI_1(u, v) \cos \varphi|^2,
\]

\((e_x, e_y, e_z)\) is the electric field in the focal region. \(\varphi\) is the angle between the plane of vibration of the incident electric field and the plane of observation. \(I_0(u, v), I_1(u, v)\) and \(I_2(u, v)\) are integrals [8] over the aperture angle \(\alpha\). \(\varphi = 0\) corresponds to the \(x\)-axis. \(u\) and \(v\) are the optical coordinates:

\[
u = nk \sin^2 \alpha z \quad \text{and} \quad r = nk \sin \alpha r,
\]

with \(r = \sqrt{x^2 + y^2}\) and \(k = 2\pi/\lambda\). \(\lambda\) is the wavelength and \(n\) the index of refraction. The axial coordinates \(u\) and \(z\) are omitted in the following since it is the resolution in the focal plane that is being investigated.

Let us assume two neighbouring light beams (a) and (b) linearly polarised in \(y\)-direction and focused to \(x_a\) and \(x_b\), respectively (fig. 1). The focal intensity distributions of beam (a) and beam (b), namely \(h_a(x-x_a, y)\) and \(h_b(x-x_b, y)\), overlap. The overlap depends on the lateral offset \(\Delta x = x_a - x_b\). Let us further assume that a fluorescent molecule in the focal region is excited by the two beams in a two-photon excitation mode so that one of the photons stems from \(h_a(x-x_a, y)\) and the other from \(h_b(x-x_b, y)\).
In this case, the excitation is governed by the product of the two neighboring intensity distributions [6]:

\[ h_{\text{ill}}(\Delta x, x, y) = h_a(x-x_a, y) h_b(x-x_b, y) \]

\[ = h_a(x, y) h_b(x-\Delta x, y). \quad (2) \]

When used in a scanning microscope \( h_{\text{ill}}(\Delta x, x, y) \) is referred to as the excitation or illumination point spread function (PSF). Since a photon from each beam is needed two-photon excitation occurs only in the region of overlap of the two beams which is determined by \( \Delta x \). Figure 2 displays \( h_{\text{ill}}(\Delta x, x, y) \) for a numerical aperture (NA) of 1.4 (oil immersion) and \( \lambda_a=\lambda_b=500 \text{ nm} \) for offsets \( \Delta x=0, 120, 200, 240, 280 \) and 300 nm, corresponding to \( \Delta \nu=0, 2.1, 3.5, 4.2, 4.9 \) and 5.3 in optical units. The PSF are calculated numerically and normalised to unity. For \( \Delta x=\Delta \nu=0 \) we have the case of normal two-photon excitation and a full-width-half maximum (fwhm) of 138 nm, or 2.43 in optical units. With increasing offset \( \Delta x(\Delta \nu) \) the excitation PSF \( h_{\text{exc}}(\Delta x, x, y) \) becomes narrower along the axis of displacement which is here the \( x \)-axis. At \( \Delta x=300 \text{ nm} (\Delta \nu=5.3) \) the fwhm is 82 nm or 1.44 in optical units. This is 59% of the fwhm of the PSF of the normal two-photon excitation imaging mode. Figure 2 reveals also that side-lobes will appear with increasing offset. At an offset of \( \Delta x=300 \text{ nm} (\Delta \nu=5.3) \) the side-lobes have a relative height of 40%. To give a feel for the effect of the offset, fig. 3 shows a surface plot of \( h_{\text{ill}}(\Delta x=0, x, y) \) in (a), and a surface plot of \( h_{\text{ill}}(\Delta x=300 \text{ nm}, x, y) \) in (b). Since \( h_{\text{ill}} \) is the product of two intensity distributions, namely \( h_a \) and \( h_b \), it is possible to generate three-dimensional images solely by illuminating the sample and collecting the fluorescence light.
However, the side-lobes would reduce the benefit of decreased lateral fwhm.

The side-lobes can be suppressed by employing point-like detection. This can be achieved by focusing the fluorescence light on a point detector, as shown in fig. 1. In this case the point spread function of the now confocally arranged scanning microscope is given by the product of $h_{\text{in}}(\Delta x, x, y)$ and the detection PSF $h_{\text{det}}(x, y)$. The detection PSF is calculated for randomly polarised fluorescence light ($\varphi = \pi/4$) and for an average fluorescence wavelength $\lambda_{\text{fluor}}$ [5]. To obtain the maximum detection signal the detector is placed so that its image in the object space is located at $(u_a + u_b)/2$. Thus, the resulting PSF is given by

$$h_{\text{conf}}(\Delta x, x, y) = h_{\text{in}}(x, y) h_{\text{det}}(x - \Delta x/2, y).$$  

(3)

Owing to the fact that the fluorescence wavelength can be nearly half the excitation wavelength $\lambda_{\text{fluor}} \approx 0.5 \lambda_a$, $\lambda_b$ the detection PSF $h_{\text{det}}(x, y)$ is narrower than the excitation PSF $h_{\text{exc}}(\Delta x, x, y)$. Therefore point detection is efficient in reducing the side-lobes.
in two-photon excitation microscopy [6]. Fig. 3c displays the confocal PSF $h_{\text{conf}}(\Delta x = 300, x, y)$ for a rather long fluorescence wavelength $\lambda_{\text{fluor}} = 0.76 \lambda_a$, $\lambda_a = 380$ nm. The height of the lobes of $h_{\text{conf}}(\Delta x = 300, x, y)$ is 3.5%.

Figure 4 displays the PSF of the (a) offset two-photon excitation confocal microscope $h_{\text{conf}}(\Delta x = 300, x, y)$ along with the (d) conventional PSF $h_{\text{det}}$ describing the lateral resolution in a conventional fluorescence microscope. For a NA of 1.4 and $\lambda_{\text{fluor}} = 380$ nm the fwhm of the PSF conventional microscope is 152 nm which compares with a fwhm of 75 nm that is achieved for a two-photon excitation at $\lambda_a = \lambda_b = 500$ nm at an offset of 300 nm and the same fluorescence wavelength. Curve (b) shows the PSF of the confocal two-photon excitation imaging mode for entirely overlapping excitation beams. The dotted curve (c) is the PSF of a single photon excitation confocal fluorescence microscope for an excitation wavelength of 488 nm and a fluorescence wavelength of 565 nm.

Figure 5a shows the decrease in the lateral extent of the confocal PSF $h_{\text{conf}}(\Delta x, x, y)$ and the excitation PSF $h_{\text{exc}}(\Delta x, x, y)$ with increasing offset $\Delta v$. Figure 5a reveals that the effect of resolution increase becomes increasingly evident at larger offsets. With increasing offset $\Delta v$, however, the region of overlap of $h_a$ and $h_b$ becomes smaller and the total probability of the two-photon excitation process decreases. This is demonstrated in fig. 5b where the decrease of the peak height of $h_{\text{exc}}$ with increasing offset $\Delta v$ is shown. For an offset of $\Delta v = 4.25$ the two photon excitation process is about 10 time less likely than without offset.
3. Set-up

The condition that the two photons stem from different beams is fulfilled when the two-photon excitation is effected by the joint action of two beams of different wavelengths $\lambda_a, \lambda_b$ when $\lambda_a \neq \lambda_b$. The utilization of light of different wavelengths excludes the possibility of two-photon excitation by a single beam. Therefore a microscope fulfilling this condition would be equipped with two different light sources, jointly focused with the appropriate offset. Two-photon excitation is possible either with or without an intermediate excited state. Ermolaev and Lyubimtsev [93] have demonstrated the stepwise excitation of fluorescent dyes, e.g. rhodamine B, to higher excited states and subsequent fluorescence emission. Here, after exciting the dye to the first singlet electronic level ($S_1 \rightarrow S_0$) with $\lambda_a$, a further excitation to a higher electronic level $S_n \rightarrow S_1$ ($n=3, 4, 5$) is effected with $\lambda_b$. The subsequent fluorescent emission from the higher states takes place in a cascade-like fashion, $S_n \rightarrow S_0$ with $n=5, 4, 3$ [9]. The fluorescence light from at least one of the higher excited states is measured, e.g. $\lambda_{fluor} \approx 380$ nm for $S_0 \rightarrow S_2$. Typical excitation wavelengths are: $\lambda_a \approx 500$ nm for $S_1 \rightarrow S_0$, $\lambda_b \geq 600$ nm for $S_1 \rightarrow S_1$. The wavelength of the second beam, $\lambda_n$, is chosen so that it is not able to effect the first transition $S_1 \rightarrow S_0$, but is still close to $\lambda_a$. A single light source can be used if it emits at least two appropriate wavelengths or if its fundamental wavelength is used in conjunction with its nonlinear derivatives.

The offset two-photon excitation with photons of the same wavelengths might be achieved with a laser emitting pulses in the femtosecond range, e.g. a modelocked Ti: sapphire laser. Each pulse is split in two separate pulses (a) and (b) and they are laterally displaced and temporally delayed in relation to each other. Pulse (a) is used for the transition $S_1 \rightarrow S_0$ and the delayed pulse (b) is used for the transition $S_n \rightarrow S_1$. The temporal delay is adjusted to be in the order of one tenth of the lifetime of $S_1$ (typically 0.1 ns) to ensure that pulse (a) has passed and that $S_1$ has not decayed. In this case a frequency-doubled mode-locked Ti: sapphire laser operating around 500 nm could be used.

4. Conclusion

By exciting a fluorophore with two photons with each photon stemming from separate, offset light beams, the extent of the PSF is reduced with respect to the extent of the normal two-photon excitation PSF (figs. 2, 5a). The resolution increase is achieved along the axis of displacement. For a lateral offset of 5.3 in optical units the fwhm of the two-photon excitation PSF is reduced to 59% of the value with no offset. Confocal detection is employed to eliminate the side-lobes and make the method suited for imaging. A fwhm of 75 nm is achieved by using visible light and conventional optics. When compared to the resolution of a conventional fluorescence microscope (fig. 4) this method offers an improvement of resolution by a factor of 2.

As the method results in a narrower PSF, the type of microscope proposed here does not require any image processing or previous knowledge of the specimen to obtain higher resolution. In contrast to the scanning optical near-field microscope it allows the observation of the interior of translucent specimens. However, the resolution improvement is achieved at the expense of the illumination intensity (fig. 5b). Sensitive detection will be required in a practical system. When applied to biological tissues care has to be taken of the higher light intensity that is needed for carrying out two-photon excitation. Pulsed lasers offering high intensities for a short duration, e.g. (fractions of) picoseconds, at a low average power might be the light source of choice.

Furthermore, I have demonstrated that the resolution-limiting effect of diffraction can be significantly overcome by carefully choosing the imaging mode of the scanning fluorescence microscope and by fully exploiting the properties of the fluorophores. Combined with modern quantum optical techniques the (confocal) scanning microscope has the potential of dramatically improving the resolution in far-field light microscopy.

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References
