Resolving fluorescence beads at 100–200 nm axial distance with a two-photon 4Pi-microscope operating in the near infrared

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Abstract

We show the possibility of resolving point objects having an axial distance of 100–200 nm with a precision of ±15 nm with a two-photon excitation 4Pi-microscope. The resolution is demonstrated by axial imaging of clustered fluorescence beads. A comparison of the axial resolution of the two-photon excitation 4Pi-microscope with a regular two-photon excitation microscope shows a four-fold improvement in axial resolution.

1. Introduction

During the past three years 4Pi-confocal microscopy has evolved as a new concept of far-field light microscopy with fundamentally increased axial resolution [12,3,5–10]. The main idea of the scanning 4Pi-confocal microscope is the coherent use of two opposite objective lenses, for illumination and/or detection of the same point in the specimen (Fig. 1). Illuminating the lenses with wave fronts interfering in the common focal region increases the illumination aperture, whereas the coherent summation of the wave fronts of the emitted light in a common point detector increases the detection aperture. Due to the confocal arrangement, the resultant PSF of the 4Pi-confocal microscope is given by the product of the illumination PSF and the detection PSF, both of which can be of the 4Pi-type [1–3]. The multiplication of the PSFs leads to an intensity-square like behaviour of the PSF. This is responsible for the axial and lateral discrimination of the 4Pi-confocal microscope and its three-dimensional imaging capability [4].

The use of two-photon excitation in 4Pi-confocal fluorescence microscopy is particularly interesting [5–7,9]. The first reason is that the illumination PSF is given by the square of the focused intensity thus leading to an intrinsic three-dimensional imaging capability of this contrast, i.e. three-dimensional imaging without confocal pinhole. The second reason is that the fluorescence wave length is almost one half of the excitation wavelength. When using a pinhole this leads to a detection PSF twice as narrow as the illumination PSF. Thus the confocal pinhole and the narrow detection PSF probes the inner part of the illumination spot, so that the overall extent of the effective (confocal) PSF is reduced [5–7,9]. Therefore, the use of a confocal pinhole also suppresses the lobes of a two-photon 4Pi illumination PSF. However, the use of a narrow detection PSF is at the expense of the amount of the detected fluorescence photons. Because the quadratic dependence on the illumination intensity inherently provides 3D imaging it is interesting to investigate non-confocal two-photon excitation 4Pi-microscopy and its applicability to high resolution imaging. Since the theory of two-photon excitation 4Pi-confocal and non-confocal
microscopy has been treated in the literature [5,7,9] we will refrain the present study to a practical case.

2. Experiments

To investigate the axial resolution of two-photon excitation 4Pi-microscopy, we prepared a sample of fluorescent latex beads (Molecular Probes, Eugene, Oregon) with a specified diameter of 100 nm. The maximum excitation wavelength of the fluorophore was 365 nm and the peak emission wavelength was 420 nm. The beads were mounted in Aquatex (Merck, Darmstadt, Germany) having an index of refraction of $1.395 \pm 0.005$ which is between water and glycerol. The sample was prepared by adding a diluted suspension of beads to the mounting medium and placing the mounting medium between two cover glasses. The sample was placed in the common focus of the two lenses of the 4Pi-microscope. In the 4Pi-microscope, one of the lenses is fixed whereas the other lens is adjusted with a precision of 10–20 nm with respect to the focus of the first lens using a three-dimensional piezoelectric stage (Lightline F 603, Physik Instrumente, Waldbronn, Germany). The position of the adjustable lens was controlled by a closed loop thus ensuring a tight common focus of the two lenses. The light source was a mode locked Ti:sapphire laser providing subpicosecond pulses at a wavelength of 750 nm. The image was recorded by scanning the sample with a second piezoelectric stage. The pixel size was 20 nm and the fluorescence light was detected with a photo multiplier working in the photon counting mode [10].

Fig. 1a displays an axial image of the sample recorded in the standard two-photon excitation fluorescence (non-confocal) mode with a single lens. The vertical axis corresponds to the optical axis (z). The size of the image is $4 \times 4$ micrometer. Fluorescence beads at different locations of the sample can be recognised. The bead images are elongated along the optical axis which is due to the axial elongation of the single lens focus. Fig. 1b shows the same place of the speci-
men but recorded in the 4Pi-mode with constructively interfering illumination wavefronts. The 4Pi axial image is clearly different because of the interference of the two focused spherical illumination wave fronts.

It is interesting to investigate the region marked with C. Fig. 2 shows the normalised axial intensity distribution along the marked axis, for the two-photon single lens and 4Pi recording. Apart from the intrinsic noise of the pixel data, the single lens curve exhibits a rather smooth decline of intensity whereas the 4Pi is governed by the interference of the two wave fronts. The main maximum has a typical full width half maximum (fwhm) of 130 ± 10 nm. The fwhm of the single lens two-photon curve is 650 ± 10 nm. A comparison of the curves with the theoretical point spread functions [6,7] show that curves in region C correspond to point spread functions (PSF), i.e. single bead images of the two-photon single lens and two-photon 4Pi-contrast, respectively. Since the main maximum and the adjacent lobes of the 4Pi-PSF are sharp along the optical axis, they can be employed for resolving axially adjacent objects. Therefore we use this experimentally gained PSF of region C for a further evaluation of the image.

The dashed line in Fig. 3 shows the normalised intensity distribution recorded along the optical axis at A, for the 4Pi-confocal microscope. The thin solid line in Fig. 4 shows the normalised intensity distribution of the same axial line but recorded in the single lens mode. Whereas we could not find any significant difference in the fwhm between region A and C in the single lens image (Fig. 4), the 4Pi axial section of Fig. 3 is clearly different from the 4Pi-PSF in Fig. 2. At the site where we expect minima, the section reveals distinct shoulders showing that the signal cannot be due to a single bead. The measured curve compares well with the calculated axial profile when assuming that we have two beads that are axially offset by 100 nm and similar intensity. The dotted line in Fig. 3 is the measured axial profile whereas the solid curve is the sum of two PSFs with an axial offset of 100 nm.

The same analysis can be performed at the region marked with B for the two-photon single lens and the 4Pi-microscope. Although, the single lens axial section in B is slightly broader (Fig. 4), the uncertainties of the curve caused by noise are too high to carry out a quantitative evaluation. In contrast to the single lens profile, the axial profile in region B gained with the two-photon 4Pi-microscope (Fig. 5) exhibits charac-
characteristic periodic peaks and valleys. Again, we find that the signal is very similar to the summation of two single bead functions of comparable intensities, separated by 170 nm. This shows that region B actually features two beads that are 170 nm apart in an axial projection. To check the precision of the separation we have varied the assumed distance. We found that the summed PSF signals significantly differed from the measured axial responses at distances equal to or larger than 190 nm and equal to and shorter than 150 nm. Furthermore, we arbitrarily varied the relative intensities of the point spread functions on the reconstruction. We found that the value of 170 ± 15 nm remained unaffected because the reconstruction did not match the measured curve for distances shorter than 155 nm or larger than 185 nm irrespective of the assumed intensities. In contrast to the two-photon 4Pi-microscope, the single lens microscope did not allow a separation of the beads in region A and B.

3. Discussion

In our evaluation we showed that the two-photon 4Pi-image contains additional information which allows the axial separation of beads at high resolution. In contrast to the single lens two-photon microscope, the 4Pi version is able to separate beads at an axial distance of 100–200 nm, and a precision of ± 15 nm. This is remarkable when considering the fact that the resolution is determined solely by the aperture and the excitation wavelength, which is here as long as 750 nm. We believe that an even more precise and reliable retrieval of object information is possible when using whole three-dimensional data sets. This remarkable precision in three-dimensional microscopy is due to the fact that the distinct pattern of minima and maxima in the 4Pi-PSF can be exploited as a "ruler" to determine the axial distances.

Since the diameter of the beads is specified with 100 nm, we interpret the beads in region A as two fluorescence beads that are stuck together. Since the diameter of the beads is comparatively large (100 nm) the beads are not fully point-like and the minima in the 4Pi-PSF cannot be totally dark. However, simple calculations considering the amount of fluorophore molecules in the bead in planes parallel to the focal plane, show that the effective fwhm of the axial molecule concentration in the beads is only about 35 nm. Therefore the beads are well enough "point-like", which is also found in the experiment.

We would like to point out that the recording of the two-photon 4Pi-image alone (Fig. 1b) already shows the places where we have bead clusters, namely those places where the 4Pi-section does not look like a regular (constructive) 4Pi-PSF, e.g. region B. The single lens recording is quite insensitive in this respect. The availability of the experimental PSF of the 4Pi-microscope facilitates the retrieval of further information about the location of the beads and is possibly the method of choice to fully analyse 4Pi images. This method does not require further assumptions about the 4Pi-PSF such as the relative phase of the interfering wavefronts. The 4Pi-PSF in Fig. 2 is not exactly constructive but this is not crucial in our case since a destructive relative phase or any other phase would have been suited for resolving the objects. The 4Pi-PSF can be translated in the image unless the index of refraction strongly varies in the sample. A strongly varying index of refraction as it is expected in fuzzy biological specimens alters the relative phase of the wavefronts and changes the 4Pi-PSF. Still, we expect that even in the case of fuzzy specimens the PSF-based evaluation will be applicable to a limited region of a few micrometers. In many cases this could be sufficient for the investigation of specific labels or distances in these regions. We would like to note that in our example the index of refraction did not match that of the oil immersion system (1.52). Aquatex has an index of refraction of 1.395 which is even lower.
than the popular glycerol. This did not affect the reconstruction of the image since the region and the distances investigated are too small for noticeable phase alterations.

When comparing the two-photon single lens and the 4Pi-image one realises that the four-fold increased axial resolution of the 4Pi-(confocal) microscope is not traded off against the lateral resolution as it is the case in the recently proposed theta-confocal microscope. The latter has a theoretically predicted axial resolution increase of about 30% when compared to a standard high numerical aperture microscope, which is gained at the expense of a 80% loss of resolution both in x and y direction, as well as a 80% loss of fluorescence light [11,12].

The advantage of the standing wave microscope [13,14] which is based on a conventional microscope and illuminates the whole sample with flat standing waves, is that it does not require scanning and gains a direct full-field image of the sample which is not the case in the 4Pi. The disadvantage of the flat standing wave microscope is that it features more, and higher lobes, and a “missing cone” in the optical transfer function in axial direction. The latter is equivalent to a lack of axial discrimination by intensity, so that the reconstruction and interpretation algorithms of the images need to be more elaborate. An evaluation by normalised intensity distributions as presented in this paper would not have been possible.

For the future it will important to develop algorithms for the fast evaluation of 4Pi-images. Such algorithms could be based on a rigorous deconvolution method [15], or on a reconstruction method with subsequent cross-correlation with the original image as employed in this paper. A promising approach is a peak function deconvolution which does not take the whole PSF for deconvolution, but a set of delta peaks that are located at the axial distance of the lobes and weighted with their height [10]. This method is applicable for two photon 4Pi-confocal images where the lobes are well below 50%. As the lobes are higher in our case, we tested a five-point peak deconvolution on the 4Pi-image the result of which is shown in Fig. 1c combined with markings of the beads at the axial distance we found with the PSF reconstruction described herein. The markings were placed at local intensity maxima we found after the five-peak deconvolution which were consistent with the axial distances found in the above described manner. The qualitative nature of this procedure urges for an improved automated deconvolution algorithm of the 4Pi images which is a further step in the development of the 4Pi-confocal microscope.

In conclusion, we have shown that two-photon 4Pi-microscopy working at an excitation wavelength of 750 nm is able to recognise different beads at 100–200 nm axial distance and to measure their distance with a precision of ± 15 nm. This is to our knowledge the first paper reporting an axial localisation and separation of distinct objects in a far-field light microscopy at such distances and precision. As the employed wavelength is in the near infrared we conclude that with a shorter wavelength the two-photon 4Pi-microscope has the capability to separate point objects at distances well below 100 nm.

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