SHORT COMMUNICATION

4Pi-confocal images with axial superresolution

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Summary

We present two-photon excitation 4Pi-confocal images of clustered fluorescence beads demonstrating threedimensional far-field light microscopy with unprecedented resolution. For an excitation wavelength of 760 nm, the lateral and axial resolution amounts to 200 and 145 nm, respectively. The four-fold improved axial resolution is achieved by engineering the point-spread function through a suitable combination of aperture enlargement, two-photon excitation, confocalization and three-point deconvolution. In contrast to their confocal counterparts, 4Pi-confocal images do not exhibit the typical axial elongation. The axial resolution in the 4Pi-confocal images corresponds to about one-fifth of the wavelength and surpasses the lateral resolution by 25%.

In the past decade, scanning far-field microscopy has experienced a rapid growth (Wilson, 1990). Recent progress in scanning fluorescence microscopy includes the development of nonlinear excitation techniques such as two-photon (Denk et al., 1990) and three-photon (Hell et al., 1996) excitation microscopy as well as new methods for increasing the resolution (Hell, 1996). Multiphoton excitation and confocal microscopes feature the unique ability to image transparent objects in three dimensions. This stems from the intrinsic axial resolution of these systems which is a result of the nonlinear dependence on the intensity of their effective point-spread function (E-PSF) (Wilson & Sheppard, 1984). This is due to the finite aperture of the focusing lens and the corresponding spatial asymmetry of the focused wavefront.

Any attempt to increase the resolution of a far-field fluorescence microscope ultimately aims at reducing the extent of the effective focus, i.e. the E-PSF. This is also the case in two-photon 4Pi-confocal microscopy (type A) (Hell, 1990; Hell & Stelzer, 1992). Here, the axial sharpening of the focus is accomplished in three steps. First, the total illumination aperture of the system is enlarged by using two opposing objective lenses of high aperture, so that the spherical wavefronts of these lenses are brought to constructive interference in the common focal point. This produces an illumination-PSF (I-PSF) with a sharp main maximum and two axial lobes on either side of the focal plane (Hell, 1990; Hell & Stelzer, 1992). By selecting the illumination wavelength appropriately, one can excite the dye in the two-photon mode and the E-PSF depends quadratically on the focal intensity (Sheppard & Kompfner, 1978). In that case, axial lobes of about 65% are present (Hell et al., 1995). One can show that even in the presence of these lobes, a two-photon 4Pi-microscope is able to resolve fluorescence beads at axial distances of 100–200 nm. However, unambiguous axial imaging is achieved only if the lobes are absent. The use of a point-like detector reduces the extent of the E-PSF in the axial direction by about 20% and further suppresses the lobes. Hence, confocalization is the second step in sharpening the focus. Finally, the lobes are entirely removed by a three-point deconvolution (Hänninen et al., 1995). As a result, a sharp monotonically decreasing E-PSF remains which is about 4–5 times narrower than that of a single lens. For an excitation wavelength of 760 nm and two lenses of 1.4 (oil immersion) the axial FWHM of the E-PSF is 145 ± 10 nm.

The resolution of a microscope can be measured by imaging clusters of point-like, or nearly point-like, objects. In our case we took fluorescent beads (Molecular Probes, #L5028) which were randomly dispersed in Aquatex® (Merck, Darmstadt). The specified diameter of the beads was...
100 nm. A small drop of the dispersion was placed on a cover slip and covered with a second slip of the same thickness. Aquatex® has a refractive index of $n = 1.39$, which is between that of glycerol and water and therefore significantly mismatched to the immersion medium which was oil. Our intention was the measurement of the resolution for a situation that is very close to many other practical cases. The beads were randomly distributed in the suspension; some of them formed clusters and others were isolated. This allowed both the determination of the PSF as well as the measurement of the separation capability of the microscopes.

Imaging was accomplished by scanning the sample in three dimensions by means of a piezoelectric scanner (Melles Griot, Cambridge, U.K.). The closed loop of these stages provided a fine scanning precision of about 10 nm. We recorded three-dimensional (3-D) data stacks both in the standard two-photon as well as in the 4Pi-confocal mode. The data stack consisted of 10–30 parallel XZ-images, and

Fig. 1. Axial image of clusters of fluorescence beads as recorded with a two-photon (a) confocal, (b, c) 4Pi-confocal microscope. In (c) the two axial lobes are removed by a three-point deconvolution.
the distance between the XZ-images in the Y-direction was set to 60 nm. The size of each image was 2 x 2.5 μm. Excitation was performed with a train of 100-fs pulses from a mode-locked Ti:sapphire laser at a wavelength of 760 nm. The numerical aperture was effectively 1.35 (oil). The light passing through a confocal pinhole was recorded with a blue-sensitive photomultiplier. The diameter of the pinhole was 10 μm, corresponding to about 40% of the area of the back-projected focal Airy disc of the fluorescence light.

Figure 1 shows a typical axial image (XZ-plane) from the 3-D data stack taken with a single lens (a), and the corresponding image from a 4Pi-confocal stack (b). To obtain a direct comparison between both methods, the single lens stack was recorded by obstructing one of the lenses of our 4Pi set-up. Thus, the 4Pi images display a higher signal-to-noise ratio. However, the most striking difference is found in the structure of the images. The image of the single lens recording is smeared out along the optical axis as a result of the lengthy shape of the standard two-photon confocal E-PSF. To emphasize this difference, we depicted the intensity profile along line 1 and line 2, which are shown in Fig. 2 for the standard (a), and the 4Pi-confocal mode (b). Figure 2(b) corresponds to a typical 4Pi-confocal axial profile of a point-object (Hell & Stelzer, 1992). The main maximum and the lobes can clearly be recognized. This is not so with line 2, which has a somewhat more complex structure. Apparently, line 1 crosses a single bead whereas line 2 crosses two beads or a bead cluster. This question can be answered by applying the three-point deconvolution for removing the lobes of the 4Pi-response. This is accomplished in Figs. 1(c) and 2(c).

The difference between the standard recording in (a) and the 4Pi in (c) is apparent. While it is hardly possible to distinguish the beads in Figs. 1(a) and 2(a), Figs. 1(c) and 2(c) provide a clear view of the object. The axial elongation of the beads has vanished. In contrast to the standard confocal microscope the 4Pi-confocal microscope resolves every single bead. In Fig. 2(c) the signal of the single bead is a sharp peak, and also each of the two beads produce a sharp peak. The resolution even allows for the determination of the distance between the beads. In line 2 the axial distance is 240 nm.

As the XZ-images are part of 3-D stacks, we also compared the stacks of the two recordings and were able to display the beads in three dimensions using a suitable computer software. This underlines the reliability of the 4Pi-technique. One can calculate that the effective dye distribution along an axis in the beads is significantly narrower (FWHM of 75 nm) than both the axial and the lateral FWHM of the E-PSF so that a bead image corresponds in first approximation to the E-PSF. We find a lateral resolution of about 200 and 140 nm in the axial direction, which is in accordance with previous measurements (Hänninen et al., 1995). In a confocal microscope of the same wavelength it is 200 nm in the lateral direction and 640 nm in the axial direction. Thus, the axial resolution is improved by a factor of 4.5. The 4Pi-confocal method neither improves nor diminishes the lateral resolution of the microscope, but it is interesting to note that the axial FWHM is about 50 nm narrower, i.e. 25% better than in the lateral direction. This is due to the fact that in a 4Pi-confocal microscope the axial aperture is more complete.

When recording 4Pi-images we ensured that the high-aperture lenses are lined up and adjusted to the common focal point with a precision of about 10–20 nm. This requirement was met by mounting one of the lenses on a piezoelectric 3-D positioning stage. In general, we favoured the following procedure. First, the second lens was aligned with respect to the first lens using one of the beads in the sample, then it was locked by the closed loop of the 3-D positioning stage. The relative phase of the spherical wavefronts was adjusted by adjusting a piezoelectrically driven mirror which was mounted in one of the illumination paths of our 4Pi-confocal microscope. The adjustment of the phase was accomplished by monitoring a 4 Pi-axial response such as the one shown in Fig. 2(b) (line 1). As mentioned above, we purposely mismatched the refractive index of the sample (n = 1.39) to the cover glass and the immersion oil (n = 1.518). Therefore, we expected the relative phase of the wavefronts to be constant only over a limited axial distance. We observed that the phase was sufficiently constant over an...
axial distance of about 2.5 μm. For our recordings, the condition of constant phase was fulfilled. For strong index mismatch, as was the case here, this effect has to be taken into account when recording large 4Pi-confocal images. It can be addressed by continuously changing the relative phase of the wavefronts when imaging stacks that are thicker than 2.5 μm. However, we would like to emphasize that this problem does not affect the resolution capability of the 4Pi-microscope since the objects that have to be separated are obviously at much closer distances, say at 100–500 nm. This should also apply to the imaging of biological specimens with varying refractive index. For a limited axial range, the 4Pi-confocal microscope should be able to resolve considerably better than a regular confocal microscope. When imaging watery specimens, the index of refraction of the mounting medium can be matched to that of the immersion sample by using high-aperture water-immersion lenses.

Figure 3 shows a further pair of axial images recorded with the two-photon (a) standard confocal and the (b) 4Pi-confocal microscope. Again, the axial image is not compromised by an elongated PSF and again the 4Pi-confocal microscope clearly resolves the bead clusters. The 4Pi-confocal fluorescence microscope differs from the standing-wave fluorescence microscope (SWM) (Bailey et al., 1993) not only by the fact that the 4Pi-confocal microscope is a scanning system. The concept of the two microscopes is different. The two-photon 4Pi-confocal microscope aims at the generation of a single sharp focus in the axial direction whereas the SWM produces a pattern of parallel interference layers. While the latter technique is simpler and possibly easier to use, it leaves in the image an uncertainty in the choice of which plane is addressed (Webb, 1995). The strength of the SWM is to provide a fast overview; the strength of the 4Pi-confocal microscope is the ability to deliver axial images similar to those of any other confocal or multiphoton fluorescence microscope. The philosophy of the 4Pi-confocal microscope is to engineer the E-PSF (Hell, 1996) of a scanning microscope. Thus we reach the classical goal of resolution increase, namely a well-defined sharper focus. The 4Pi-confocal axial images presented in this paper are, to our knowledge, the best resolved images taken with a far-field fluorescence microscope, thus accounting for a major progress in the endeavour for substantially increased far-field resolution.

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References


