Measurement of the 4Pi-confocal point spread function proves 75 nm axial resolution

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In a 4Pi-confocal microscope the specimen is illuminated and observed coherently from above and below such that the numerical aperture is increased [S. W. Hell, European Patent Application 91121368.4 (filed 1990, published 1992), S. W. Hell and E. H. K. Stelzer, J. Opt. Soc. Am. A **9**, 2159 (1992)]. The point spread functions of 4Pi-confocal and confocal microscopes were measured. Our measurements prove a three- to seven-fold increase of axial resolution, thus opening the prospect for a powerful three-dimensional imaging technique with an axial resolution down to 75 nm.

In far-field light microscopy, imaging is accomplished by focusing elements. An objective lens focuses light by concentrating a segment of a spherical wave front into an object point. The intensity in the focal region is distributed around the focal point forming a focal volume, which is described by the point spread function (PSF). The extent of the PSF determines the resolution in a far-field microscope.[1] When no aberrations are present the PSF is determined only by the wavelength of the light in the object medium and the aperture angle of the lens. The extent of the PSF decreases with decreasing wavelength and with increasing aperture angle. Since the use of wavelengths below 360 nm is limited by the performance of the optical components, the resolution is further improved by increasing the aperture angle.

The largest theoretical aperture angle is 4π, that of a spherical wave front. This is, however, hardly achievable in a practical system. But the aperture angle of a light microscope can be increased by using two opposing lenses on either side of the sample with a common focal point (Fig. 1). A confocal microscope is an excellent basis to realize such an increase in aperture angle since in a confocal microscope point-like illumination and point-like detection are applied.[2] The wave fronts originating from the point-like light source are split into two coherent parts that illuminate each lens separately. Similarly, the wave fronts collected by each lens are directed to a single point detector. This concept is realized in the 4Pi-confocal microscope[3] (Fig. 2). Since confocality yields an effective PSF, that is, the product of the illumination and the detection PSFs,[2,4] resolution improvement is achieved if the extent of at least one of the PSFs is reduced.[3] The conditions for reducing the extent of the illumination PSF are the illumination wave fronts are coherent and interfere in the common focus. The conditions for reducing the extent of the detection PSF are the collected wave fronts are coherent and interfere in the point detector. Obstructing one of the lenses transforms a 4Pi-confocal microscope to an ordinary confocal microscope.

Axial sections of the PSF of a confocal and a 4Pi-confocal arrangement in which both the illumination and the detection PSFs are improved were measured. Oil immersion lenses with numerical apertures of 1.4 (Zeiss 100× Planapochromat) were used. A He-Ne laser operating at a wavelength of 633 nm was employed. The sample consisted of gold beads of 30 nm diam (colloidal gold, Janssen Products, Olen, Belgium) immersed in immersion oil and mounted between two cover slips. In both experiments a single bead was scanned in a plane containing the optical axis and the scattered light intensity was recorded as a function of the position. Scanning was performed by a scan stage that offered independently controlled x, y, and z axes (Photon Control, Cambridge, England). The bead was scanned 1.5 μm along the optical axis and 0.8 μm in lateral direction. The smallest resolvable step of the stage was 10 nm, thus providing a high-precision measurement of the PSF.

The confocal resolution was determined by using the 4Pi-confocal arrangement with the lower path obstructed. Figure 3(a) shows the intensity of the scattered light as a

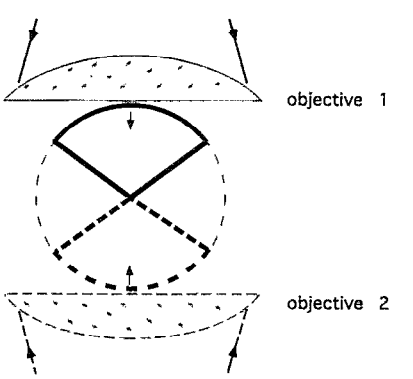

FIG. 1. In conventional far-field light microscopy only a segment of a spherical wave front is focused into or collected from an object point. A higher spatial resolution is achieved when not only a segment but a complete spherical wavefront is used. Thus, focusing or collecting is improved when a second lens provides another segment of the complete spherical wave front thus increasing the angles of the focused and/or collected wave fronts.

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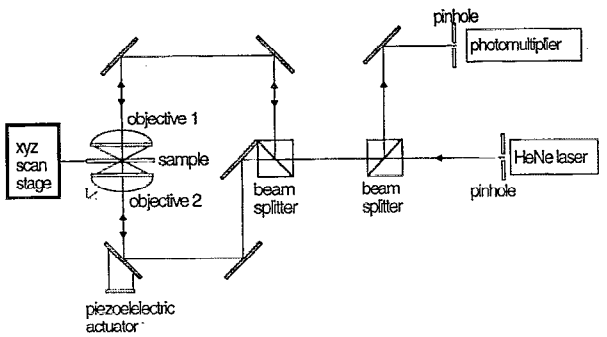

FIG. 2. The 4Pi-confocal microscope. Two high aperture lenses with a common focus are used to increase the aperture of illumination and collection. To achieve the common focus one of the lenses is adjusted with a piezoelectric stage (Physik Instrumente, Waldbronn, Germany). The wave front coming from the illumination pinhole is spit with a beam splitter to illuminate each of the lenses. The light collected from both lenses is combined with the same beam splitter and directed toward the detection pinhole. The piezoelectrically actuated mirror allows the adjustment of the relative phase of the wave fronts. The 4Pi-confocal microscope acts as a confocal microscope when one of the lenses is obstructed. The 4Pi-confocal microscope can be used in a scattering light mode when polarized light is used for illumination and a crossed polarizer is placed in front of the detector.

function of the lateral (r) and axial (z) directions. It presents an axial section of the point spread function of a confocal microscope operating at a wavelength of 633 nm using a lens with a numerical aperture of 1.4 (oil). The full width at half-maximum (FWHM) is 200 nm in lateral direction and 520 nm in axial direction. The FWHM are indicators for lateral and axial resolution. The point spread function is cylindrically symmetric around the optical axis.

To determine the resolution of a 4Pi-confocal microscope, the same experiment was carried out, but without the obstruction in the lower path. Two lenses were used simultaneously for illumination and detection. Illumination was performed with linearly polarized light. A polarizer oriented perpendicular to the polarization of the laser light was placed in front of the detector preventing transmitted light from entering the detector. Thus, only the light scattered by the bead was detected. The central part of the entrance pupil of both lenses was obstructed with a circular beam stop having a third of the diameter of the entrance pupil of the objective lenses. The difference in length of the upper and lower path (2 mm) was smaller than the coherence length of the laser light so that the partial wave fronts were able to interfere in the focus and in the point detector.

Figure 3(b) shows the axial section of the 4Pi-confocal microscope's point spread function in the case of constructive interference. Compared to the confocal case, the main maximum is greatly reduced in its axial extent: The axial FWHM is 75 nm which is seven times smaller, thus promising a sevenfold increase in (axial) point resolution. The lateral FWHM is 160 nm. This slight increase in lateral resolution can be attributed to the central obstruction of the

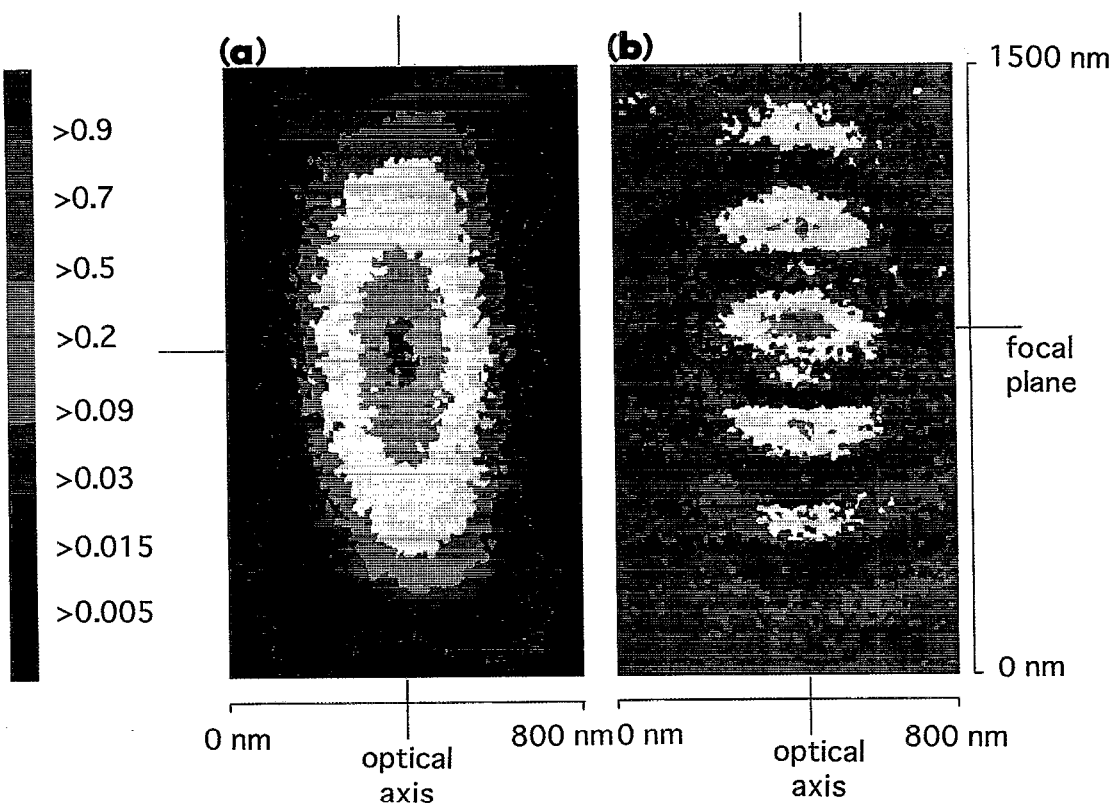

FIG. 3. Axial section of the point spread function of a confocal microscope (a) and of a 4Pi-confocal (b) microscope with a numerical aperture of 1.4 (oil) at a wavelength of 633 nm in a plane containing the optical axis. The point spread function is cylindrically symmetric around the optical axis. It is normalized to unity. The shading shows the intensity drop with increasing distance from the focal point. The axial full width at half-maximum of the main maximum is 520 nm in the confocal and 75 nm in the 4Pi-confocal case. Its lateral extent is 200 nm in (a) and 160 nm in (b). The smaller focal volume in (b) is due to the increased aperture for illumination *and* detection.

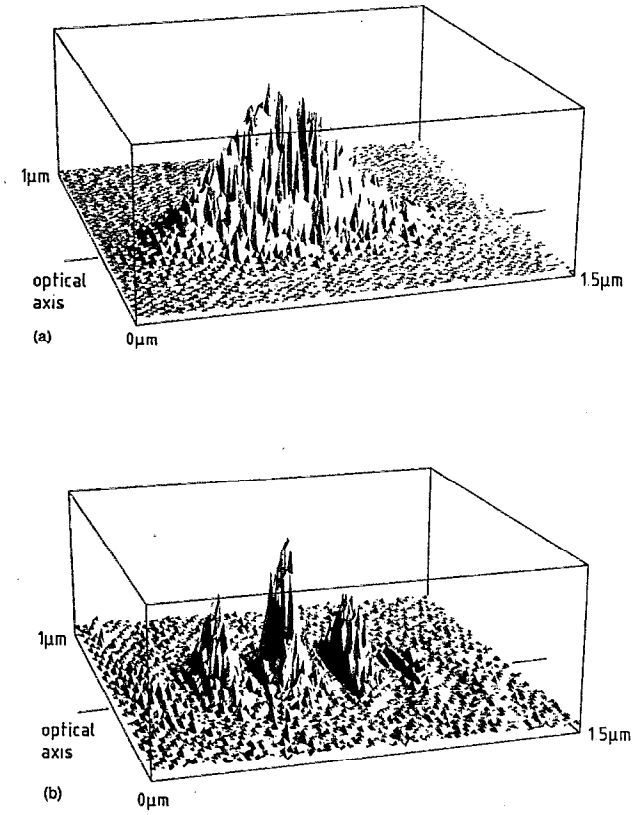

FIG. 4. Surface plot of the axial sections of the (a) confocal, (b) 4Pi-confocal point spread functions emphasizing the seven times narrower main maximum in (b) and the axial lobes. The height of the axial lobes is 58% of that of the main maximum.

aperture. The two axial lobes are due to the fact that not an entire spherical wave front is used. The height of the lobes is 58% of that of the main maximum. They stem from the fact that the focused/collected wave fronts do not form an entire sphere. A deconvolution is required to get rid of the axial lobes. Since this process depends on the signal-to-noise ratio of the data set a minimal improvement of resolution of 3 (the decrease of the volume of the PSF over that in a confocal microscope) and a maximal improvement of 7 can be expected. Figure 4 shows the surface plot of (a) the confocal and (b) the 4Pi-confocal PSF emphasizing the narrow main maximum of the latter and its axial lobes. The presence of the lobes reduces the benefit of a decreased width of the main maximum. The 4Pi-confocal resolution enhancement in *fluorescence* microscopy was shown elsewhere.[3] The use of two-photon fluorescence excitation or of a fluorescent dye with a long Stokes shift reduces the height of the axial lobes in a 4Pi-confocal fluorescence microscope.[5]

Employing two lenses of high numerical aperture to coherently illuminate an object point and to coherently collect the light scattered by an object increases the axial resolution in far-field light microscopy. The resolution can be further increased when a lower wavelength is used. For a wavelength of 350 nm, an axial FWHM of 40 nm can be expected. We believe that 4Pi-confocal microscopy has a potential to become *the* technique for high resolution light microscopy.

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[3] S. Hell and E. H. K. Stelzer, J. Opt. Soc. Am. A **9**, 2159 (1992).
[4] S. Hell, E. Lehtonen, and E. H. K. Stelzer, in *Visualization in Biomedical Microscopies,* edited by A. Kriete (Verlag Chemie, Weinheim, 1992), p. 145.
[5] S. Hell and E. H. K. Stelzer, Opt. Commun. **93**, 277 (1992).