## Confocal microscopy with an increased detection aperture: type-B 4Pi confocal microscopy

Stefan W. Hell and Ernst H. K. Stelzer

Light Microscopy Group, European Molecular Biology Laboratory, Postfach 10 22 09, D-69012 Heidelberg, Germany

## Steffen Lindek and Christoph Cremer

Institut für Angewandte Physik, Universität Heidelberg, Albert-Überle-Strasse 3-5, D-69120 Heidelberg, Germany

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We show the point-spread function of a confocal microscope with an increased detection aperture. The increase in aperture is accomplished by coherent collection of the light from the specimen with two opposing objective lenses, i.e., type-B 4Pi confocal microscopy. We demonstrate experimentally its feasibility for detecting scattered or fluorescently emitted light. The 4Pi confocal point-spread functions are shown for constructive and destructive interference of the collected wave fronts and are compared with the point-spread functions of comparable confocal microscopes.

The major advantage of the confocal microscope is its axial discrimination that permits optical sectioning of the sample.<sup>1</sup> Axial discrimination is equivalent to axial resolution, which is quantified by the extent of the intensity point-spread function (PSF) along the optical axis. Therefore a reduction of the extent of the PSF is important for the optical-sectioning properties of the microscope. We can obtain this reduction either by decreasing the wavelengths used for illumination and for detection or by increasing the numerical aperture.

4Pi confocal microscopy was proposed as a means to increase the aperture angle and therefore improve the axial resolution of a confocal microscope.<sup>2-4</sup> Since in a confocal arrangement the PSF is given by the product of the illumination and the detection PSF's, three types of 4Pi confocal microscope have been described. In a type-A 4Pi confocal microscope the illumination aperture is enlarged, whereas in a type-B 4Pi confocal [4Pi(B) confocal] microscope the detection aperture is increased. A type-C 4Pi confocal microscope combines both types A and B, leading to further resolution enhancement along the optical axis. The potential of axial resolution with a type-A 4Pi confocal microscope was shown by measurement of the z response of a fluorescent layer.<sup>3</sup>

In this Letter we show the effect of an increased detection aperture on the resolution through the use of 4Pi(B) confocal microscopy. First, we present PSF's obtained by recording the light scattered by nanometer-scale particles. Second, we show z responses recorded with a fluorescent sample.

Figure 1 shows the 4Pi(B) confocal microscope used for the experiments with scattered light. We produced a pointlike light source by focusing a laser beam into a pinhole. The light leaving the pinhole was collimated by a lens (L1) and then split into two beams by a beam splitter (BS2). One beam was focused into the sample by a microscope objective (OBJ1), and the other was blocked with a polarizer oriented perpendicular to the polarization of the laser light. A second objective (OBJ2) was placed on the other side of the sample and focused into the focal point of the first objective. The light coming from the sample was collected on both sides through the objective lenses. The two detection beams were deflected from the illumination path by a beam splitter (BS1) and focused into the detection pinhole by a lens (L2). Another crossed polarizer placed in front of the photomultiplier tube prevented light with the original polarization from being detected.

Coherent detection of the light requires spatial and temporal coherence of the detected light. Temporal coherence is guaranteed by a path-length difference smaller than the coherence length of the detected light. Spatial coherence is an inherent property of the confocal microscope.<sup>1</sup> Therefore the wave fronts coming from the opposing objectives interfere in the detector, provided that the detection paths are nearly equal. Depending on the relative phase, the interference can be constructive or destructive. From the quantum-mechanics point of view the scattered or fluorescently emitted photon is able to propagate along both paths simultaneously.



Fig. 1. Setup of the 4Pi(B) confocal microscope: M's, mirrors; PH's, pinholes; PMT, photomultiplier tube. Two opposing objective lenses are used to detect coherently the scattered light emanating from the sample.

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In our experiments the path-length difference was adjusted with a piezoelectrically driven mirror in the lower path. The increase in detection aperture was realized by two oil-immersion microscope objectives, both with a numerical aperture of 1.4 (Zeiss  $100 \times$  Planapochromat). The 633-nm light of a He-Ne laser was used for illumination. The sample consisted of 30-nm Au beads (colloidal Au, Janssen Products, Olen, Belgium) immersed in oil and mounted between two cover slides. Oil immersion avoided refraction-index mismatch between the sample and the immersion medium. The sample was scanned through the focal region with a piezoelectrically driven scan stage (Photon Control, Cambridge, UK) that provided independent movement along all three axes with an accuracy of 10 nm.

Figure 2(a) shows the confocal PSF; Figs. 2(b) and 2(c) show the 4Pi(B) confocal PSF's obtained with this setup. The represented plane contains the optical axis. The PSF is cylindrically symmetrical around the optical axis. It is normalized to unity in all three cases. In order to record a confocal PSF we blocked the lower path of the 4Pi(B) confocal microscope, and illumination and detection were performed with the upper objective (OBJ1). The confocal PSF [Fig. 2(a)] has an axial FWHM of 510 nm and a lateral FWHM of 185 nm. The 4Pi(B) confocal PSF [Fig. 2(b)] exhibits an interference pattern with constructive interference. In other words, the light coming from the geometric focal point interferes constructively in the point detector. The main maximum has FWHM's of 120 nm (axially) and 200 nm (laterally). The heights of the axial lobes are 0.65 and 0.55. The 4Pi(B) confocal PSF [Fig. 2(c)] was obtained with a path-length difference increased by half the wavelength of that in Fig. 2(b) and therefore exhibits an interference pattern with destructive interference. In other words, the light coming from the geometrical focus interferes destructively in the detector.

In a similar experiment the detection aperture was increased for a confocal fluorescence microscope. The following changes were made to the setup in Fig. 1: The beam splitter (BS1) that was used to separate the detection path from the illumination path was replaced by a dichroic mirror that deflected the fluorescence light into the detection pinhole. The polarizers in the lower path and in front of the detector were replaced by long-pass filters that blocked the 633-nm laser light but transmitted the fluorescence light, e.g., RG645 or RG665 filters (Schott, Mainz, Germany). The sample consisted of a more than  $10-\mu$ m-thick fluorescent layer of a Nile Blue A perchlorate solution (Kodak Optical Products, CAS no. 53340-16-2). This layer was mounted between two cover slides oriented perpendicular to the optical axis and scanned along the optical axis while the fluorescence signal of the glass-fluorophore edge was recorded. The first derivative of this signal is the z response to an infinitely thin layer and quantifies the axial resolution of planes. Figure 3 presents the results of the fluorescence experiments with a confocal microscope [Fig. 3(a)] and a 4Pi(B) confocal microscope for constructive [Fig. 3(b)] and destructive [Fig. 3(c)] interference. The confocal PSF [Fig. 3(a)] has a FWHM of 800 nm. In the case of the 4Pi(B) microscope with constructive interference [Fig. 3(b)] the central maximum has a FWHM of 225 nm, but the axial lobes reach heights of 0.73 and 0.76.

Both experiments show that in 4Pi confocal microscopy the minima between the main maximum



Fig. 2. Surface plot of an axial section of the PSF of (a) a confocal and (b), (c) a 4Pi(B) confocal microscope by use of scattered light. Shown is an x-z plane containing the optical axis. All PSF's are normalized. In (b) the path-length difference is a multiple of the wavelength, whereas in (c) it is an odd multiple of half the wavelength. The confocal axial FWHM of 510 nm (a) is reduced to 120 nm for the main maximum in 4Pi(B) confocal microscopy (b). The axial lobes in (b) reach heights of 0.65 and 0.55.

![](_page_2_Figure_1.jpeg)

Fig. 3. The z response for (a) the confocal and (b), (c) the 4Pi(B) confocal fluorescence microscope. All z responses are normalized. In (b) the path-length difference in a multiple of the wavelength, whereas in (c) it is an odd multiple of half the wavelength. We obtained the z responses by scanning a fluorescent layer along the optical axis; these responses represent the laterally integrated PSF. The central maximum in 4Pi(B) confocal microscopy has an axial FWHM of 225 nm (b). The axial lobes in (b) reach heights of 0.73 and 0.76.

and the first axial lobes have values between 0.3 and 0.4. This is not the case in type-A 4Pi confocal microscopy, in which the minima are nearly zero.<sup>3</sup> Provided that there is a high degree of coherence, minima with heights of 0.3 correspond to an intensity ratio of 12 for the two detection beams. When one is working with scattered light, the beam splitter (BS2) and the polarizer in the lower detection path can be considered to be responsible for an intensity ratio of 3 or 4, with the lower beam being weaker than the upper one. The Mie effect,<sup>5</sup> which is responsible for a stronger scattering in the forward direction than in the backward direction, strengthens the lower beam, thus reducing or even reversing the intensity ratio that is due to the beam-splitting and filtering elements. However, a factor of 12 cannot be explained by these effects, and it can be excluded that the heights of the minima are due to a large difference between the amplitudes of the detected light in the two paths. A similar assessment can be made for the fluorescence case. The reasons for the heights of the minima are currently under investigation.

4Pi(B) confocal microscopy offers the prospect of resolution enhancement, provided that it is possible to reduce the axial lobes and to benefit fully from the decreased axial FWHM. This can be done, for example, by use of fluorescent dyes that can be excited with a wavelength that is much smaller than the fluorescence wavelength. In this case the lobe values of the detection PSF are multiplied by much smaller values of the illumination PSF, and thus the axial lobes are suppressed.<sup>6</sup> This reduction of the illumination-to-detection wavelength ratio is feasible with dyes, e.g., lanthanide chelates, that have a large Stokes shift. Possible fluorophores are europium chelates, which can be excited by 365-nm light with an Ar-ion laser working in the UV range and have a fluorescence emission at 614 nm.

We show what are to our knowledge the first experiments studying the effects of an enhanced coherent detection aperture in light microscopy. The results of our experiments differ from the results for type-A 4Pi confocal microscopy in that our results do not have minima that are nearly zero. 4Pi(B) confocal microscopy has the potential for increasing the axial resolution, especially if combined with fluorophores that have a large Stokes shift.

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