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Brakenhoff, G.J.; Müller, M.

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Improved axial resolution by point-spread autocorrelation function imaging

G. J. Brakenhoff and M. Müller

BioCentrum Amsterdam, Department of Molecular Cytology, University of Amsterdam, Kruislaan 316,
1098 SM Amsterdam, The Netherlands

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Based on an interferometric spatial autocorrelation of two shifted point-spread functions combined with confocal detection, we demonstrate that the point-spread autocorrelation function technique provides improved axial resolution compared with conventional confocal imaging. The principle of the technique is demonstrated for a one-sided fluorescing step object. © 1996 Optical Society of America

In recent years several approaches have been developed to improve the resolution of the far-field light microscope. Confocal microscopy¹⁻⁴ provides increased resolution and (most importantly) optical sectioning, which permit the imaging of three-dimensional objects within a specimen with diffraction-limited resolution. The effective numerical aperture (NA) of the microscope can be increased by use of more than one objective, as is demonstrated in 4π microscopy^{5,6} and theta microscopy.⁷ Alternatively, the conventional resolution limit can be extended by use of advanced schemes to modify the excitation point-spread function (PSF) (e.g., see Refs. 8 and 9).

In this Letter we present a new technique for resolution improvement beyond the confocal limit in far-field microscopy that is based on the confocal detection of the response of a specimen to two correlated illumination distributions. Here the point-spread autocorrelation function (PSAF) presented earlier¹⁰ is used for imaging, offering the advantage of being inherently robust because it relies on a common path interference arrangement. Furthermore, it requires no special specimen preparation, can be used with any conventional fluorescing dye, and can be incorporated in a relatively simple manner within a standard confocal microscope.

As discussed in detail elsewhere,¹⁰ the PSAF technique is based on the interferometric correlation of two illumination distributions at the focal point of a high-NA lens, which one accomplishes by periodically varying the relative phase of one of the illumination beams with respect to the other and detecting the (oscillating) fluorescence signal. The two illumination distributions can be shifted spatially with respect to each other, and the fluorescence signal is detected confocally. Denoting the two (complex) illumination distributions u_{ref} and u_{obj} (with the subscripts referring to the reference and the object fields, respectively), we can write the generating function for the PSAF imaging signal as

$$G(\Delta\mathbf{r}, \Delta\phi) = \int_{-\infty}^{\infty} d\mathbf{r} \mathcal{O}(\mathbf{r}) \cdot |u_{\text{ref}}(\mathbf{r}, \phi_0) + u_{\text{obj}}(\mathbf{r} + \Delta\mathbf{r}, \phi_0 + \Delta\phi)|^2 \cdot |u_{\text{det}}(\mathbf{r} + \frac{1}{2}\Delta\mathbf{r}, \phi_0)|^2. \quad (1)$$

In Eq. (1) the spatial shift between the reference and the object beams is denoted $\Delta\mathbf{r}$, $\Delta\phi$ denotes the phase shift relative to an arbitrary initial phase ϕ_0 , and $\mathcal{O}(\mathbf{r})$ is the object function that describes the spatial structure of the fluorescing sample. The confocal detection is described by the intensity PSF of the imaging system $|u_{\text{det}}(\mathbf{r} + \frac{1}{2}\Delta\mathbf{r}, \phi_0)|^2$ and is positioned midway between the reference and the object PSF's. Note that in principle the illumination and the detection PSF's will be different ($u_{\text{ref}} = u_{\text{obj}} \neq u_{\text{det}}$). For small Stokes shifts between excitation and emission wavelengths, however, they are almost identical. The detected PSAF signal is given by

$$I_{\text{PSAF}}(\Delta\mathbf{r}) = \text{Max}\{G(\Delta\mathbf{r}, \Delta\phi); \Delta\phi \in [0, 2\pi]\} - \text{Min}\{G(\Delta\mathbf{r}, \Delta\phi); \Delta\phi \in [0, 2\pi]\}, \quad (2)$$

where Max() and Min() denote, respectively, the maximum and minimum detected fluorescence intensity when the relative phase of the object beam is shifted with respect to the reference beam over the interval $0-2\pi$.

The experimental setup for the PSAF measurements is depicted schematically in Fig. 1. The 0.1-mW output of an air-cooled argon-ion laser (Spectra-Physics) at 488 nm is collimated and enlarged by a telescope to a full width at half-maximum (FWHM) of ~ 8 mm to provide proper filling of the full aperture of the objective. At this power level no effects of either bleaching or saturation are observed, which may affect the performance of the system.¹¹ The beam is split into two parts, the reference and the object, by a 50% beam splitter (BS₁). The two beams are recombined on a second 50% beam splitter (BS₂), reflected off a dichroic mirror, and focused onto a fluorescing sample by a Leitz 63/1.30–0.60 oil objective. The fluorescence, centered at 555 nm, is detected in the backscattering direction through an OG530 blocking filter and a confocally placed 10- μm pinhole with a photomultiplier tube. A sine-wave-driven piezo element provides 120-Hz modulation on the relative phase of the object beam with respect to the reference beam. The amplitude of the modulation is exactly one wavelength, i.e., induces a phase shift of $0-2\pi$, and we can

Fig. 1. Schematic of the experimental setup: T, telescope; L_{ref} , L_{obj} , 200-mm lenses; BS₁, BS₂, 50% beam splitters; L, 100-mm lens to provide proper tube lens conditions; D, dichroic; O, objective; S, sample on a variable height translation stage; BF, blocking filter; P, 10- μ m pinhole; PMT's, photomultiplier tubes; DS, digital sampling scope; LIA, lock-in amplifier.

adjust the relative offset between the two beams by varying the dc offset on the piezo element. The lenses (L_{ref} and L_{obj}) can be translated to provide an axial shift between the two illumination distributions. We accomplish phase-sensitive detection of the PSF signal by using the second recombined beam from BS₂ as the reference signal for a lock-in amplifier (PAR Model 121). The output of the lock-in amplifier is digitized on a digital oscilloscope (LeCroy 9361). The sample consists of a 10^{-3} M Rhodamine 6G solution in water contained in a 200- μ m capillary (Vitro Dynamics). The axial position of the sample can be adjusted with a stepper motor control with a precision of ~ 0.1 μ m and is measured with a capacitive sensor (Queensgate Model LS50).

At the zero position of lenses L_{ref} and L_{obj} the focal points of the reference and the object beams coincide with each other and with the conjugate detection distribution of the confocal pinhole. The size of the pinhole (10 μ m) is chosen well below the size of the (diffraction-limited) first Airy ring in the illumination distribution multiplied by the lateral magnification, to provide maximum confocal sectioning. Special care has been taken to ensure that L_{ref} and L_{obj} translate exactly along the optical axis to avoid lateral displacement of the PSF's during the axial movement of the lenses.

The results of the measurements of the axial PSF signal in the bulk of the fluorescing sample (approximately 5 μ m below the interface) are shown in Fig. 2. Here the amplitude of the fluorescence oscillations that are due to the periodic phase change induced by the piezo element is measured as a function of the axial focal shift (Δz) between the reference and the object illumination PSF's. In all that follows, the axial focal shift is expressed in units of wavelength in vacuum λ_0 ($= 488$ nm). The illumination PSF's are shifted symmetrically with respect to the detection PSF; i.e., when one is shifted to a position above the

focal plane the other is shifted by an equal amount to a position below the focal plane. Note that the confocal detection PSF is stationary and is always positioned halfway between the two illumination PSF's. Just as for the lateral case,¹⁰ the axial PSF signal is functionally similar to the axial PSF, showing nodes and side-lobe structure. Also, the width of the axial PSF has a strong square dependence on the effective NA of the objective (not shown), which we controlled by reducing the diameter of the illumination beam with an iris.

The improvement of axial resolution through PSF imaging is demonstrated in Fig. 3, where the PSF response to a fluorescing step object is shown for a number of axial focal shifts (Δz) between the object and the reference PSF's. We obtain the step response by moving the interface of the fluorescing sample through focus. The data show that some fringes introduced by the axial focal shift are not fully suppressed. For $\Delta z = 0$ the PSF response is equal to the conventional confocal fluorescence response, as shown in Fig. 3(b) by the open-circle data point. As a rule of the thumb the axial distance between the 25% and 75% levels of the response to a step function can be taken as a measure for the FWHM of the axial (confocal) PSF.¹² This FWHM can be calculated to be ≈ 0.6 μ m for an immersion oil objective ($n = 1.51$, NA 1.3, $\lambda_0 = 488$ nm). Figure 3(b) shows that our experimental result of 0.95 μ m at $\Delta z = 0$ is close to this theoretical value. The error bars denote the accuracy with which the steepness can be determined from the data and with which the focal shift between the reference and the object PSF's can be adjusted.

As can be seen from Fig. 3, the observed PSF response at the focal shift $\Delta z = 0$ is equal to the measured (conventional) confocal fluorescence response. We observe a steepening of the PSF response to the step object with increasing focal shifts, demonstrating the resolution improvement that can be realized in the PSF technique. An important point is that the observed confocal fluorescence response and the PSF response at $\Delta z = 0$ are $\sim 50\%$ less step than what can be expected from theory, i.e., 1.0 rather than 0.6 μ m. A similar discrepancy in experiments with high-NA lenses has been reported.^{11,13} The causes for this discrepancy may be various, e.g., prediction errors

Fig. 2. Axial PSF signal as a function of NA. The dashed lines are baselines for the individual curves.

Fig. 3. (a) PSFA response to a fluorescing step object for various focal shifts between the reference and the object PSF's. The dashed lines are baselines for the individual curves. The relative decrease in the actual signal levels is 1/0.5/0.2 for focal shifts 0/2.1/3.1 λ_0 . (b) The steepness, expressed as the distance between the 25% and 75% amplitude levels, of the axial PSFA response to a fluorescing step object as a function of the focal shift between the reference and the object PSF's. The open-circle data point represents the conventional confocal fluorescence response. Note that the zero separation is suppressed.

owing to approximations in the theoretical model, non-ideal lens properties, and aberrations. However, our results clearly demonstrate that with the PSFA tech-

nique a resolution improvement over the confocal response of $\sim 30\%$ can be obtained even in the presence of such aberrations.

When considering imaging with the PSFA signal one must realize that there is in fact not one unique PSFA image but rather a family PSFA images, with each image collected at a certain focal shift between the two generating field distributions. Although it was demonstrated here only for the axial case, the focal shift can in principle be extended to all three dimensions.

We have demonstrated the possibility of increased axial resolution of PSFA imaging over that which can be obtained with confocal imaging. The technique employs a common path interferometric arrangement that is inherently robust. Also, any specimen used in conventional fluorescence microscopy can be used, and no major modifications of the microscope are required. The experiments presented here, as well as those for the lateral PSFA, confirm the resolution improvement of PSFA imaging over that of confocal imaging expected from our theoretical modeling. Based on these calculations, it is found that an improvement of the axial resolution is expected also for the case of two thin fluorescing layers. In this case the positions of two planes could be resolved, according to the Rayleigh criterion, for a separation distance between the two planes of $\sim 30\%$ less than that required in conventional confocal imaging, when PSFA imaging with an appropriate axial focal shift is used.

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