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Apodization and the point-spread autocorrelation function

M. Müller and G. J. Brakenhoff

A novel function, the point-spread autocorrelation function (PSAF), which is closely related to the point-spread function, of a high numerical aperture microscope objective is introduced. The function is both experimentally measured and theoretically modeled for various apodization conditions. These include varying the effective numerical aperture of the objective, applying annuli of different size, and illuminating the objective with a spatially nonuniform intensity distribution. An excellent agreement between experimental data and theoretical modeling is obtained without the use of any fitting parameters. The PSAF technique is sensitive to the various apodization conditions, affecting both the width of the PSAF signal and the amplitude of the sidelobes. A potential use of the technique is the measurement of the effective numerical aperture and the optimization of the illumination conditions in complex microscopical systems. © 1997 Optical Society of America

Key words: Point-spread function, apodization.

1. Introduction

The performance of a high-resolution optical imaging system, such as the laser scanning confocal microscope,^{1,2} is determined by both the quality of the imaging system itself and its illumination conditions. The term illumination conditions refers to the amplitude and phase distribution of the incident optical field over the aperture of the imaging system. In a practical microscope, many factors may reduce the total performance of the system: a slight misalignment of the illumination beam may cause vignetting or asymmetries in the illumination profile; the buildup of dirt or exposure to shock may decrease the quality of the optical system gradually; the numerical aperture (NA) of the objective may be overestimated by the manufacturer³; or an annular amplitude filter may be incorporated in the objective to make the image look crispier.

To first order, these effects can be viewed as a modification of the amplitude distribution over the aperture of the objective. This in turn will modify the point-spread function (PSF) of the system, generally

leading to an increased width of the distribution—that is, a change in the effective NA of the system as a whole—and a change in the position and relative magnitude of the sidelobes.

The intensity PSF can be determined from imaging the fluorescence or scattered light from a small object with a moving slit or pinhole in front of the detector.^{1,4–6} Although three-dimensional focal intensity distributions can be measured accurately with this technique, it suffers from relatively slow data acquisition, high demands on the long-term stability of the optical setup, and special sample preparation. These drawbacks render these techniques less than adequate for routine evaluation and optimization of complex imaging systems.

Recently we developed a new technique⁷ to determine the point-spread autocorrelation function (PSAF), which yields a response similar to the intensity PSF. This technique is fast, accurate, and relatively simple. Furthermore, there are no limiting constraints on either the illumination source or the sample, so an evaluation and optimization of the optical system can be done with test conditions that closely match those in which the experimental data are obtained.

Here we present the results of experimental measurements and theoretical modeling of the PSAF signal for a high-NA system subjected to various apodization conditions. In a slight variation of the commonly used definition of the term,^{8,9} we use the following restricted definition of the term apodization

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Fig. 1. Schematic of the experimental setup for PSAF measurements: BS's, 50% beamsplitters; L_1 , 200-mm lens; L_2 , 80-mm lens; PAR, lock-in amplifier to rectify the PMT output.

in this paper: the modification of the amplitude profile over the aperture of the lens, without altering the plane-wave phase front. Changes in the effective NA of the optical system are readily observed, and the technique is sensitive to various apodization conditions, such as the application of annuli or an asymmetric illumination profile. In Section 2 we introduce the principle of the PSAF technique and the experimental setup to measure this signal. Section 3 describes the procedure followed for theoretical modeling of the data based on a numerical evaluation of scalar diffraction theory. In Section 4 we present the results of the experiments and the theoretical modeling for various apodization conditions, and Section 5 concludes the paper with a discussion of these results.

2. Point-Spread Autocorrelation Function

A. Function Definition and Experimental Setup

The generating function for the PSAF technique is given by the interferometric spatial autocorrelation of the focal optical field of a (high numerical aperture) lens:

$$G(\Delta \mathbf{r}, \tau) = \int_{-\infty}^{\infty} dt \int_{-\infty}^{\infty} d\mathbf{r} \mathcal{O}(\mathbf{r}) |u(\mathbf{r}, t) + u(\mathbf{r} + \Delta \mathbf{r}, t + \tau)|^2. \quad (1)$$

In Eq. (1), $u(\mathbf{r}, t)$ denotes the (three-dimensional) focal optical field in space and time behind the aperture stop of the lens; $\Delta \mathbf{r}$ represents a spatial shift, τ represents a temporal delay, $|\cdot|^2$ represents the complex square, and $\mathcal{O}(\mathbf{r})$ represents the object function specifying the spatial structure of the object. Because we assume the object to be of limited extent, Eq. (1) will necessarily converge.

Figure 1 shows the experimental setup, which includes the different aspects of the principle of the PSAF technique as described by Eq. (1). The 0.1-mW output of an air-cooled cw argon-ion laser, tuned to a wavelength of 488 nm, is let to expand by

natural divergence to a beam with a FWHM of approximately 8 mm. It then passes a 6-mm-diameter diaphragm to provide approximately a top-hat distribution of power, before being split by a 50% beam splitter (BS_1) into two parts. For convenience one part is called the reference in the following, whereas the other is called the object. The object beam passes a variable delay line, which introduces a temporal delay τ , before being recombined on a second 50% beam splitter (BS_2). This second beam splitter is connected to a motorized tilt control over one axis of orientation, perpendicular to the axis of propagation of the beam. It is placed telecentrically with respect to the objective to ensure tilt independent aperture filling. The driving voltage of the motorized tilt control, with a frequency of 0.1 Hz, is digitized on one channel of a digital oscilloscope. The thickness of the beam splitters (6.4 mm) ensures that the interferometric setup is not affected by ghost reflections.

After the second beam splitter, the recombined beams are focused by a 200-mm lens (L_1) at a point located in accordance with the tube length of the objective of which the focal field distribution is to be measured. A three-element compound objective (Leitz oil plan 100, NA = 1.25) is used in the experiments. The objective focuses the beams into a sample, which consists of a 10^{-4} M solution of Rhodamine 6G in water, contained in a 0.1-mm microslide. The induced fluorescence is detected in the backscattering direction, through a dichroic mirror and an OG530 blocking filter, by a photomultiplier tube (PMT). The fluorescence is imaged, with a demagnification factor of 2, onto the PMT with an 80-mm lens (L_2). The PMT is also placed telecentrically with respect to the objective aperture.

Tilting the recombining beam splitter introduces a (lateral) spatial shift $\Delta \mathbf{r}$ of the object focal field, which is denoted by $u(\mathbf{r} + \Delta \mathbf{r}, t + \tau)$ in Eq. (1), with respect to that of the reference, which is denoted by $u(\mathbf{r}, t)$ in Eq. (1). The absorption of radiation by the fluorophores in the dye solution serves as a local quadratic detector, which is denoted by the complex square, $|\cdot|^2$, in Eq. (1). Because in these experiments a homogeneous dye solution is used, the object function $\mathcal{O}(\mathbf{r}) = 1$ for all $|\mathbf{r}|$ smaller than the spatial extent of the microslide. The primary signal results from the measurement of the time-averaged fluorescence intensity, integrated over the focal extent of the optical field, as a function of both the focal shift $\Delta \mathbf{r}$ and the temporal delay τ .

There are in principle many possibilities for measuring and analyzing the primary signal obtained from Eq. (1). However, an especially straightforward and robust implementation of the above-described principle and the one employed in these experiments uses an additional periodic movement of the variable delay line by mounting it on a home-built shaker. Typically a shaker frequency of 19.4 Hz with an amplitude of approximately $66 \mu\text{m}$ is used. The periodic variation in optical path length of the object beam induces, through interference, oscilla-

tions in the fluorescence intensity at a frequency determined by the wavelength of the laser and the frequency and amplitude of the shaker (at approximately 5.2 kHz for the setup used). The amplitude of these oscillations is measured by frequency filtering and rectifying the PMT output with a lock-in amplifier and is subsequently digitized on a second channel of the oscilloscope. The PSAF signal is given by the amplitude of these oscillations as a function of the induced focal shift between the reference and object focal field:

$$I_{\text{PSAF}}(\Delta \mathbf{r}) = \max\{G(\Delta \mathbf{r}, \tau); \tau \in [0, \lambda/c]\} - \min\{G(\Delta \mathbf{r}, \tau); \tau \in [0, \lambda/c]\}, \quad (2)$$

where $\max()$ and $\min()$ denote the maximum and minimum of the total, space-integrated fluorescence intensity, detected for temporal delays τ between the object and reference focal field, within the range of zero to λ/c (a relative phase shift between zero and 2π). For the signal-to-noise ratio to be improved, all measurements presented in this paper have been averaged over five subsequent scans of the induced lateral focal shift.

In all data presented, the lateral focal shift of the object focal field with respect to the reference focal field is given in nanometers. Calibration of the induced lateral focal shift to the applied voltage on the motorized tilt control of the second beam splitter is measured in the following way. A 100- μm pinhole, with a micrometer-control lateral translation stage, is placed at the primary focal point of the 200-mm lens (L_1), that is, in a plane conjugate with the object plane. For different voltages applied to the tilt control, the position of the pinhole for maximum throughput of the laser light is determined. Taking into account that a 100 $\times$ objective is used in the measurements presented in this paper, the functional dependence of the induced lateral focal shift on the applied voltage is determined. Simultaneously with the measurement of the amplitude of the fluorescence oscillations, the voltage applied on the motorized tilt control is digitized by the oscilloscope. The recorded voltage is subsequently scaled to nanometers of induced lateral focal shift. Because of the limited accuracy of this calibration procedure, there is an uncertainty of approximately 5% in the value for the lateral focal shift. More accurate procedures to calibrate the lateral focal shift are currently under investigation. Within the experimental precision of the measurements, no influence was observed from either changing the polarization of the incident optical field or rotating the objective around the optical axis.

B. Physical Model of the PSAF

As a help toward a basic qualitative understanding why the magnitude of the PSAF signal depends critically on the shape of the intensity PSF, despite the space integration over the fluorescence from a bulk dye solution, the lateral amplitude and phase distribution of the optical field in the geometrical focal

Fig. 2. Amplitude and phase of the (complex) focal field distribution, as a function of the lateral coordinate in the geometrical focal plane, of a high-NA lens. The focal field distribution is calculated for a circular aperture and $\text{NA} = 1.3$.

plane are plotted in Fig. 2. At every node in the amplitude of the PSF there is a π phase jump. When the PSF's of the reference and object focal fields coincide ($\Delta \mathbf{r} = 0$), there is a perfect (identical) phase relation between the two fields over the total spatial extent of the focal region. Hence, when a phase shift between zero and 2π , that is, a temporal delay between zero and λ/c , is imposed on the object field, the (space-integrated) fluorescence signal will vary between total cancellation and maximum intensity as a result of interference. When, however, the PSF of the object focal field is laterally shifted with respect to the reference focal field ($\Delta \mathbf{r} \neq 0$), there will be zones along the lateral coordinate where, at temporal delay $\tau = 0$, the phase difference between the object and reference fields is zero, and there will be other zones where this phase difference is π . Thus, with increasing shift the magnitude of the difference between the maximum and minimum total fluorescence for various interference conditions, induced by varying the temporal delay, will diminish through an increasing cancellation of the contributions of the various zones. The lateral positions found at which full cancellation of the different contributions occurs turns out to be close but not identical to the zero positions in the usual intensity PSF (see Section 3). The picture becomes increasingly complex when planes are considered at positions along the z axis farther away from the geometrical focal point. Here the phase and amplitude of the focal field distribution vary more rapidly with the lateral coordinate. However, the principle of the increasing and decreasing cancellation of the contributions of the various zones, as a function of the lateral focal shift, still holds.

3. Theoretical Modeling

As a starting point for a theoretical modeling of the PSAF, we chose a numerically calculated intensity PSF. To this end the three-dimensional focal field distribution of a high numerical aperture lens system was calculated from numerical integration of the first Rayleigh–Sommerfeld integral in the Kirchhoff approximation by using the SSP (Stamnes–Spjelkavik–Pedersen) method,¹⁰ which provides an algorithm

Fig. 3. (a) Typical functional dependence of the intensity PSF and PSAF for equal focusing conditions: $NA = 1.3$ and a circular aperture. The inset is an enlargement showing that the nodes of the PSAF are at lateral focal shifts close but not equal to the lateral positions of the nodes of the intensity PSF. (b) The (space-integrated) value of the fluorescence as a function of the lateral focal shift for various values of the induced temporal delay. At a certain lateral focal shift all fluorescence values for the different temporal delays coincide, implying a zero magnitude in the difference between the maximum and minimum values of the total fluorescence. The focusing conditions are the same as in (a).

that is both fast and accurate. The focal intensity distribution was found from the complex square of the focal field distribution. The algorithm converged rapidly, requiring less than 100 integration steps in each integration coordinate. For simplicity, we applied scalar diffraction theory, thus neglecting any effects of polarization.

Following Eqs. (1) and (2), the reference focal field, which is not subjected to either a focal shift or a temporal delay, is calculated as the focal field distribution centered at the geometrical focal point of a high-NA lens. The lateral focal shift of the object focal field induced by the recombining beam splitter is modeled by the shift invariance approximation, that is, the focal field at any point near the on-axis geometrical focus is assumed to be equal to the on-axis focal field distribution except for a spatial translation. The temporal delay introduced by the variable delay line is in turn modeled by introducing an appropriate phase shift to the object focal field. The PSAF signal, at every value for the lateral focal shift, is calculated from Eq. (2) for a number of tem-

Fig. 4. Comparison of the experimental PSAF signal with the theoretically expected PSAF response for three different values of the NA of the objective: (a) $NA = 1.3$, (b) $NA = 0.9$, (c) $NA = 0.6$. The experimental data represent an average over five subsequent scans of the induced lateral focal shift. The theoretical curves are calculated for the given NA and excitation wavelength without any fitting parameters.

poral delays, corresponding to phase shifts between zero and 2π . The magnitude of the PSAF signal is taken as the difference between the maximum and minimum values obtained over this phase interval. We tested the validity of the shift invariance approximation by numerically calculating the focal field distribution for several off-axis positions and comparing it with the on-axis distributions. Within the accuracy of the calculation, no significant deviations were observed for the lateral focal shifts applied.

A typical result of the calculation of the PSAF signal is given in Fig. 3(a). In this calculation a circular aperture with $NA = 1.3$ is used. Because a circular aperture, an on-axis geometrical focal point, and scalar diffraction theory are used in the theoretical modeling, rotation symmetry with respect to the axis of propagation applies to the focal field distribution. In this case we refer to the lateral coordinate as the distance from the axis of propagation in the plane

Fig. 5. PSAF signal for different sizes of annuli, with (a) 0%, (b) 16.7%, (c) 50%, (d) 66.7% of the central part of the aperture (NA = 1.3) blocked. The experimental data represent an average over five subsequent scans of the induced lateral focal shift. The theoretical curves are calculated for the given NA and excitation wavelength without any fitting parameters.

perpendicular to it. For comparison, the intensity PSF, calculated at an equal NA, is shown in the same figure. Clearly there is an apparent similarity in the shape of the two functions. This includes the existence of sidelobes and the position of the nodes. The inset, which is an enlargement of Fig. 3(a), shows that the nodes for the PSAF are found at lateral focal shifts that are close but not identical to the lateral position of the nodes of the intensity PSF. The fact that the PSAF equals zero at certain phase shifts is demonstrated in Fig. 3(b). Here the (space-integrated) value of the fluorescence is plotted as a function of the lateral focal shift for various values of the induced temporal delay. At a certain lateral focal shift, all fluorescence values for the different temporal delays coincide, that is, they are independent of the induced phase shift, implying that the magnitude of the difference between the maximum and minimum values is zero.

4. Experimental Results

The experimental data presented below are a demonstration of the dependence of the PSAF signal on various apodization conditions. To this effect, the functional dependence of the PSAF signal on the effective NA of a high-NA objective and on the presence of annuli in the illumination path of this objective is measured. Also the sensitivity of the PSAF technique to variations in the intensity distribution over the aperture of a high-NA objective is examined.

A. Numerical Aperture

The NA of a lens is defined by $NA = n \sin \theta$, where n is the refractive index of the medium through which the light propagates from the lens to the focal point and θ is the half-aperture angle of the lens. The position of the first node in the intensity PSF along the lateral coordinate in the focal plane depends to a good approximation on the NA of the lens as

$$r_{\text{lat}} = 0.61\lambda/NA. \quad (3)$$

Hence with a decreasing effective numerical aperture, the lateral, as well as the axial, width of the focal field distribution increases.

As confirmation of the theoretical expectation that the PSAF signal shows a functional dependence on the effective NA similar to the intensity PSF, the measured PSAF signal is shown in Fig. 4 for different values of the effective NA. The effective NA of the objective is changed by varying the diameter of the diaphragm before the first beam splitter (see Fig. 1). These experiments show that the PSAF signal indeed broadens with decreasing NA. The sidelobes, that is, the Airy rings characteristic of the focal field distribution of a high-NA objective, also show up in the PSAF signal. The theoretical curves are calculated without any fitting parameters, using only the NA of the objective and the wavelength of the laser as input parameters. The slight discrepancy of the exact position and amplitude of the nodes for the highest NA is probably due to one of two causes: the illumina-

tion of the objective is not constant, which results in an effective decrease of the aperture of the objective, or else the first Rayleigh–Sommerfeld integral is inaccurate at a high NA.⁹ This latter explanation agrees with the experimental observation that with an increasing width of the PSAF signal, the agreement between theory and experiment, especially with respect to the amplitude of the sidelobes and the position of the nodes, improves.

B. Apodization Conditions

For a demonstration of the sensitivity to different apodization conditions, and a further experimental check, of the PSAF technique, a series of measurements was undertaken by using different annuli. The lateral width of a focal field distribution can be reduced at the expense of the axial width by using an annulus in the pupil plane.^{1,2,11,12} With the introduction of an annulus the relative intensity of the (lateral) sidelobes of the focal field distribution increases. The results of the measurement of the PSAF signal for different sizes of annuli are shown in Fig. 5. In all parts shown, the circular numerical aperture of the objective was the same: $NA = 1.3$. The size of the central (also circular) obstruction area changed from zero to 66.7% of the full aperture. Both the theoretically expected reduction of the lateral width and the increase of the intensity of the sidelobes, relative to the central peak, are clearly demonstrated in Figs. 5(a)–5(d). The slight asymmetry in the sidelobe structure in Figs. 5(c) and 5(d) is due to a limitation in the scanning range of the tilt of the recombining beam splitter. Note that, again, no fitting parameters have been used in the theoretical calculation of the PSAF signal.

In Fig. 6 the effect of displacing the illumination profile of the objective, with respect to the optical axis, on the PSAF signal is shown. The displacement is expressed as the ratio of the shift of the center of the illumination distribution with respect to the full aperture of the objective. The direction of the displacement is perpendicular to the optical axis and is in the same direction in which the object beam is scanned with respect to the reference beam. Displacing the illumination profile in effect introduces a spatially nonuniform distribution of the illumination intensity across the aperture of the objective. This in turn reduces the effective NA of the system and results in a broadening of the PSAF signal. Also, because an increasing amount of energy is blocked by the aperture, the amplitude of the signal decreases. Note also that the node and sidelobe structure becomes increasingly less pronounced with an increasing displacement of the illumination distribution with respect to the center of the aperture.

In another experiment, the 50% annulus of Fig. 5(c) was displaced, within the (stationary) illumination distribution over the aperture of the objective, with respect to the optical axis. When the annulus is displaced different parts of the aperture of the objective are used, while the illumination profile is virtually unchanged. The results of these experiments

Fig. 6. (a) Geometry of the displacement of the illumination profile across the aperture of the objective with respect to the optical axis. The displacement is in the same direction as the object beam is shifted with respect to the reference and is expressed as the ratio of the shift of the center of the illumination distribution with respect to the full aperture of the objective. (b) Experimental PSAF signal for several displacements varying from zero to 50%. The experimental data represent an average over five subsequent scans of the induced lateral focal shift. (c) Drop in intensity of the PSAF signal at a zero lateral focal shift as a function of the displacement.

are shown in Fig. 7. The displacement is expressed as the ratio of the shift of the center of the annulus with respect to the full aperture of the objective. The direction of the displacement of the annulus is perpendicular to the optical axis and is in the same direction in which the object beam is scanned with respect to the reference beam. Because the displacements are small compared with those of Fig. 6, no significant effect can be expected of a slightly changing intensity gradient across the illumination distribution. The dramatic effect on the node and sidelobe structure of the PSAF signal is thus almost completely due to applying an annulus asymmetrically with respect to the optical axis.

5. Discussion

In this paper we presented experimental data that demonstrate the sensitivity of the PSAF technique to various apodization conditions of a high-NA lens. The good agreement between the experimental data and the theoretically calculated curves, without the

Fig. 7. (a) Geometry of the displacement of an annulus within the (stationary) illumination profile across the aperture of the objective with respect to the optical axis. The displacement is in the same direction as the object beam is shifted with respect to the reference and is expressed as the ratio of the shift of the center of the annulus with respect to the full aperture of the objective. The experimental PSAF signal is shown for several displacements of the 50% annulus, at (b) 0%, (c) 3.5%, (d) 6.5%. The experimental data represent an average over five subsequent scans of the induced lateral focal shift.

use of any fitting parameters, as shown in Figs. 4 and 5, supports the validity of the theoretical model. The theoretical modeling may be further improved by applying the Debye approximation and incorporating the vectorial properties of the optical fields. Improvements in the experimental setup, both with respect to the calibration of the lateral focal shift and the sensitivity of the detection, are currently being developed.

The properties of the PSAF technique have been demonstrated primarily by its dependence on the effective NA of a high-NA objective and on spatially nonuniform intensity distributions over the aperture of the objective. With decreasing NA the width of the PSAF signal increases, and its shape, including the sidelobe structure and the position of the nodes, is similar to the intensity PSF. The sidelobe structure becomes more pronounced when different annuli are employed. With a spatially nonuniform intensity distribution across the aperture of the lens, the width of the PSAF signal as a function of induced lateral focal shift increases, as a result of an effective decrease of the NA. This effect is accompanied by a disappearance of the sidelobe structure. In case of the use of annuli, the disappearance of the sidelobe structure is much more pronounced. One of the unique properties of the PSAF technique, when compared with PSF measurement techniques, is that all these effects can be measured in the bulk of a dye solution and without any kind of confocal detection scheme.

In high-resolution microscopy, small nonuniformities in the intensity distribution over the aperture of the objective may readily occur. These may be due to, for instance, slight misalignment, vignetting, or differences in transmission and reflection along the illumination path for rays with different inclinations to the optical axis. These changes in apodization affect the overall performance of the instrument. Because the PSAF technique is sensitive to apodization, it may be used for the optimization of such systems or for the tracking down of possible causes of degraded performance. For instance, to check the uniformity of the illumination distribution over the aperture of an objective, the PSAF signal can be recorded with an annulus centered on the aperture. Only if the illumination profile is symmetric with respect to the annulus will maximum modulation be observed in the sidelobe structure of the PSAF signal. Also, we may record the PSAF signal as a function of NA. With increasing NA possible aberrations in the system will cause the experimental signal to depart from the theoretically predicted one.

In conclusion, the PSAF technique provides a fast and simple way to measure the effective NA of a focusing system. It is very sensitive to asymmetric illumination intensity distributions over the aperture of the objective, especially when applied in combination with an annulus. Because the PSAF technique can be used with almost any (point) light source, solvent, and dye, it may be applied as a tool for the

alignment of complex optical systems, such as high-resolution light microscopes.

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