Single sharp spot in fluorescence microscopy of two opposing lenses

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We demonstrate theoretically, experimentally, and in an imaging application the possibility to generate a single predominant sharp diffraction maximum in the effective point-spread function of a fluorescence microscope that coherently uses two opposing lenses. This is achieved through binary pupil filters that preclude the origination of the unfavorable strong interference side maxima that are otherwise present in these systems. Mathematical postprocessing, which has so far been a prerequisite to gain artifact-free images, is now optional or obsolete. © 2001 American Institute of Physics. [DOI: 10.1063/1.1407303]

In scanning confocal and multiphoton microscopes, imaging is performed with a spatially confined, three-dimensional (3D) effective point-spread function (EPSF), the extent of which determines the spatial resolution. Therefore, the most direct way of improving the resolution is to define methods reducing the EPSF spatial extent. Due to the laws of diffraction, the minimal full width at half maximum (FWHM) of the main maximum of the EPSF is of the order of 200 nm in the transverse direction, but only 500–600 nm along the optic axis. This discrepancy clearly calls for methods rendering an axially narrower EPSF.

If we leave aside the concept of stimulated emission depletion microscopy, which requires two trains of ultrafast light pulses, the only way to axially sharpen the FWHM three- to sevenfold is by interfering wave fronts of two opposing lenses, as it is utilized in scanning 4Pi-confocal microscopy, a) as well as in the nonscanning standing-wave 3D microscopy. Unfortunately, this approach is challenged by the fact that the resulting narrower main maximum is accompanied by interference sidelobes causing periodic artifacts in the image. A remedy for the lobes is data deconvolution; however, this is applicable only if the optical transfer function (OTF) is contiguous at the given noise level. In spite of reported advancements, the ultimate goal of creating a single clear-cut spot that is much sharper than in standard microscopy has not been achieved with these systems.

We now report that by applying amplitude filters in a 4Pi-confocal microscope employing coherent illumination for two-photon excitation (type A), a solitary maximum can be produced, that is 4–5 times sharper than its confocal counterpart. This solitary maximum is accompanied by only minimal lobes. As a result, the need for computational post-processing of the acquired data, which up to now has been critical, is virtually eliminated. The structure of most objects can now be inferred from the raw image data. Equally important is the fact that through the absence of computation, lower signals can be tolerated in the image.

Consisting of a single dark ring (DR), the amplitude filter is very simple (Fig. 1). Since we insert it only in the illumination path no fluorescence light is wasted. Similar filters were proposed for axial super-resolution in confocal microscopy, b) but the predicted axial narrowing is only of the order of 15%, and hence, only had little impact in practice. This is not so if two opposing lenses are used since any small reduction of the axial width of the main maximum leads to a sharp decline in the height of the interference sidelobes.

In the way it works, the filter can be perceived as a minimal Toraldo filter; however, in our case it is not devised for reducing the FWHM of the central peak, but for canceling the energy in the interference sidelobes. The filter simply divides the illumination wave front of the two lenses into an inner part and an outer ring, each producing a standing wave. While the standing wave from the inner part has a high spatial frequency, the one produced by the outer ring features a frequency reduced by the cosine of the angle between the marginal rays of the aperture and the optic axis, which is of the order of 0.4. Upon coherent overlap the main maximum is strengthened, but the field of the first side maxima, having opposing signs, is reduced. As a result, the energy of the first side maxima is spread across their higher-order counterparts, which can now be suppressed by multiphoton excitation and confocalization.

This reasoning is fully supported by the results shown in

FIG. 1. Scheme of the dark ring (DR) filter inserted into the two arms of a 4Pi-confocal microscope of type A with coherent illumination.
Fig. 2, displaying the measured and calculated $z$ responses of a $4Pi$-confocal microscope, that is, the curve obtained through scanning an infinitely thin fluorescence plane along the optic axis:

$$ I(z) = C \int_0^\infty \int_0^{2\pi} \left[ E_1(z, r, \phi) + E_2(-z, r, -\phi) \right]^{2n} \times \left[ h_{\det}(z, r) \otimes d(r) \right] r dr d\phi. $$

The field of the two lenses is calculated through

$$ E_i(u, v, \phi) = \left[ -iA_1(1_0 + 1_2 \cos 2\phi), -iA_1 \sin 2\phi, -2AI_1 \cos \phi \right], $$

with $I_{0,1,2}$ being numerically calculated integrals over the aperture angle $\theta \in [0, \alpha_{\text{max}}]$ as defined in Ref. 8, but for a pupil function taking into account the ring filter:

$$ F(\theta) = \begin{cases} 
1 & \theta \leq \alpha_1; \alpha_2 \leq \theta \leq \alpha_{\text{max}} \\
0 & \alpha_1 < \theta < \alpha_2
\end{cases}. $$

The angles $\alpha_1$ and $\alpha_2$ are defined by the inner and outer radii of the ring, $r_1$ and $r_2$, respectively. The detection PSF $h_{\det}(z, r)$ is calculated for a single lens field, using the appropriate fluorescence wavelength and convolved with the image function $d(r)$ of the confocal detector pinhole in the focal plane, which is 1 for $r < D$ and 0 elsewhere. The parameter $n$ defines the mode of excitation, with $n = 2$ denoting two-photon absorption.

The solid line in Fig. 2 shows the calculated $z$ response $I(z)$ for $\alpha_1 = 0.25$, $\alpha_2 = 0.82$, and $\alpha_{\text{max}} = 1.1$, corresponding to a numerical aperture of 1.35 oil immersion, a refractive index of 1.518, $D = 167 \text{ nm}$, $\lambda_{\text{exc}} = 760 \text{ nm}$, $\lambda_{\text{det}} = 580 \text{ nm}$, and $n = 2$. As intuitively anticipated, the $z$ response is characterized by a strong main maximum with a FWHM of 120 nm, i.e., ~4 times narrower than its confocal counterpart. Importantly, the lobes are suppressed to values of 14%, which in many applications should be close to the noise level.

To examine whether this solitary maximum can be realized, we measured the corresponding $z$ response in a $4Pi$ setup (Fig. 1). For this purpose, the beam of a mode-locked Ti:Sapphire laser was divided into two partial wave fronts, each of which passed a fused silica plate of $\lambda/10$ planarity. An aluminum ring was glued onto each plate, serving as the binary dark ring filter. The wave fronts propagated to the lenses (PL APO Leica, 100×, 1.4 NA oil) to interfere at the common focal point. The radii of the rings were $r_1 = 0.8 \text{ mm}$ and $r_2 = 2.35 \text{ mm}$, yielding the $\alpha_{1,2}$ elected above.

The fluorescence light was collected through one of the lenses without obstruction. After passing through a dichroic mirror and a Schott BG39 color glass filter, it was focused onto a detector pinhole of diameter 0.65 times the Airy disk. The $z$ response is obtained by scanning a monomolecular, fluorescent Langmuir–Blodgett film along the optic axis.

The theory is impressively confirmed by the measurement (Fig. 2). Similarly to the calculation, the FWHM of the main maximum of the experimental $z$ response is 120 nm and the interference sidelobes are down to 14% of the central peak, which is 2.5 times lower than the lobe height obtained without amplitude filter. Comparison with the standard (two-photon) confocal $z$ response with FWHM 550 nm demonstrates that the DR-4Pi-confocal microscope provides right away with a fundamentally sharper effective PSF, testifying to a higher axial resolution.

In the optical transfer functions of two-lens systems, the presence of lobes is manifested as dents ($4Pi$), strong depressions ($I^2M$), or even zeros (SWM) within the enlarged bandwidth of the OTF.\textsuperscript{5,9} Depending on the noise level, the weak regions of the OTF render the required data processing challenging ($I^2M$) or even impossible (SWM). The sharp focal peak of Fig. 2, however, yields an enlarged axial OTF without periodic dents. This is demonstrated in Fig. 3, which depicts the Fourier transform of the $z$ response for the confocal and the DR-4Pi-confocal microscope. The comparison shows that our scheme not only enlarges the axial bandwidth but also strongly transfers spatial frequencies throughout the region of support of the OTF. This greatly facilitates image postprocessing, which, however, is not always mandatory in this system.

To prove this, we recorded Escherichia coli bacteria both with the standard confocal setting and with its DR-4Pi-confocal counterpart. For this purpose the bacterial membranes were labeled with the dye DH-5α and mounted in Aquatex (Merck, Inc.). The membrane basically forms a hollow “bag,” which is too small to be resolved by the

FIG. 2. $z$ responses of the DR-4Pi microscope feature a pronounced main maximum and low sidelobes, found in the calculation (solid) and in the measurement (boxes). The broad curve (diamonds) exhibits the standard confocal $z$ response, whereas the crosses the $4Pi$ $z$ response without DR. The inset shows the computed $z$ response for three-photon excitation. Note the excellent agreement between theory and experiment and the marked improvement of axial resolution.

FIG. 3. Measured optical transfer function along the reciprocal optic axis of the confocal (light solid) and the DR-4Pi-microscope (solid) featuring a fundamentally enlarged bandwidth.
As can be noticed from the theoretical and experimental data, as well as in the images, the reshuffling of the intensity to the outer lobes leaves a small haze in the raw data. By applying a three-photon excitation process in a DR-4Pi-confocal microscope, say at $\lambda_{exc}=1000\text{nm}$, the haze would be reduced since in this case the sidelobes are readily reduced to 3.3%, as shown in the inset of Fig. 2. However, as a three-photon process leads to restrictions as far as the mode of excitation is concerned, we think that two other approaches will be more successful. First, one could apply a mathematical deconvolution, which because of the favorable OTF should be very effective; in many cases, it is a simple linear mathematical filter. Second, one could refine the dark ring filter. As is the case also in the Toraldo concept, one could devise filters with more than just one ring and filters featuring smooth amplitude and also phase transitions. Clearly, such filters will also be helpful in the single-photon excitation mode.

We have calculated the radial structure of the EPSF of the DR-4Pi microscope and found that the lateral side maxima are increased only slightly, and that the overall lateral resolution is not decreased. The reason for the first is the strong confocal suppression, whereas for the second it is the annular structure of the DR filter, which is even known to lead to lateral super-resolution.

In summary, we have demonstrated theoretically and experimentally a predominant focal peak in a fluorescence microscope using two opposing lenses that is 4.6 times sharper than that of confocal microscopy. Our approach uses simple aperture filters in the illumination path of the objective lenses and leaves a solitary main focal maximum. As demonstrated in the images, fundamentally superior axial resolution in 3D microscopy can be achieved without mathematical postprocessing.

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