4Pi microscopy with linear fluorescence excitation

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Practical 4Pi microscopy has so far exclusively relied on multiphoton excitation of fluorescence, because the nonlinear suppression of contributions from higher-order sidelobes was mandatory for unambiguous axial superresolution. We show that novel lenses of 74° semiaperture angle enable biological 4Pi microscopy with regular one-photon fluorescence excitation, thus increasing the signal and reducing system complexity and cost. An axial resolution of 95 nm, corresponding to a more than fourfold improvement over confocal microscopy, is verified in the imaging of microtubules in mammalian cells. © 2007 Optical Society of America

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4Pi microscopy has improved the axial resolution in fluorescence microscopy by a factor of 3–7 over its conventional or confocal counterpart. Relying on the constructive coherent addition of the wavefront caps of opposing high-angle lenses, the intensity point-spread function (PSF) of this microscope features a narrow main diffraction maximum that is accompanied by higher-order side maxima (axial lobes) above and below the focal plane. The effect of the side maxima has to be removed by image processing for 4Pi microscopy to provide artifact-free 3D images. As a rule of thumb, this mathematical removal is possible if the first-order axial sidelobes are <50% of the central maximum. The sidelobe issue is even more severe in the related wide-field approach of IM microscopy, in which only the wavefront caps of fluorescence emission are added.

Multiphoton excitation greatly helps in reducing the sidelobe artifacts in two ways. First, the higher-order dependence of the excitation on the illumination intensity decreases the fluorescence from the lower sidelobes with respect to that from the main diffraction maximum. Second, for a 4Pi configuration of type C, i.e., simultaneous coherent addition of the illumination and fluorescence wavefronts, the disparity between the multiphoton excitation and fluorescence wavelength causes the lobes of the illumination and detection PSF to peak at different points in space. Since the effective PSF is a product of the two, the lobes cancel out each other, whereas the main peak is reinforced. However, limitations of the multiphoton strategy are that it usually requires expensive mode-locked lasers and that it is usually accompanied by stronger bleaching. Thus, the use of one-photon excitation in 4Pi microscopy would not only reduce complexity and cost but also lead to brighter images. Moreover, the shorter excitation wavelength used for one-photon excitation should provide a narrower main maximum of the effective 4Pi PSF, not only along the z axis but also in the focal plane.

Several strategies have been proposed to render 4Pi microscopy feasible with one-photon excitation, such as the suppression of the fluorescence from the lobes by stimulated emission and the use of dedicated pupil filters. Unfortunately, none of them can be readily integrated into existing systems. In this Letter, we solve the problem by building on the very premises of the concept, that is, on the increase of the solid aperture angle of the system. As a result, we demonstrate the feasibility of 4Pi fluorescence microscopy with one-photon excitation, allowing reliable 3D imaging in cells with an axial resolution of 95 nm.

As invoked by the name, 4Pi microscopy and also IM rely on large-angle spherical wavefronts. So far the largest semiaperture angle available in (flatfield) well-corrected immersion lenses was 68°. Recent developments in lens manufacturing have allowed a significantly larger angle, α = 74° (Objective HCX PL APO CS 100/1.46 Oil, Leica Microsystems, Mannheim). The calculation of the PSF shows that the increase in angle by 6° suppresses the lobes by 7% in the intensity PSF of two opposing lenses (Fig. 1). A confocalized one-photon-excitation 4Pi microscope of type C features an effective PSF that is given by

\[ h_{\text{eff}}(r) = h_{\text{exc}}^{4\Pi} \cdot h_{\text{det}}^{4\Pi} = |E_{\text{exc}}(r) + \hat{M}E_{\text{exc}}(\hat{M}r)|^2 \cdot |E_{\text{det}}(r) + \hat{M}E_{\text{det}}(\hat{M}r)|^2 \cdot p(r), \]

where \( E_{\text{exc}} \) and \( E_{\text{det}} \) are the focal fields for illumination and detection, respectively. \( \hat{M} \) is a transformation matrix accounting for the counterpropagation of the spherical wavefronts, and \( p(r) \) denotes the pinhole function.

Figure 1 displays the numerical results for the z response of the system \( I_z(z) = \int \int dxdy h_{\text{eff}}(r) \), i.e., the normalized signal generated by an ultrathin horizontal plane, for NA=n sin α equal to 1.40 and 1.46 oil immersion, corresponding to α of 68° and 74°, respectively, along with NA=1.20 water immersion. The refractive indices are \( n=1.51 \) (oil) and \( n=1.33 \) (water), whereas the excitation and fluorescence wavelengths...
were assumed to be 488 and 605 nm, respectively. The Fourier transformation of \( I_z(H_{20849}) \) yields the optical transfer function (OTF) along the inverse optical axis \( k_z \). High lobes in the spatial domain imply an attenuation of the OTF at the critical spatial frequency \( k_c \) corresponding to the distance between the maxima. The effect of the latter can be removed only if \( \text{OTF}(k_z) \) is not swamped by noise.

Figure 1 shows that even the small increase in aperture angle by 6° decreases the sidelobes by 7%, thus elevating \( \text{OTF}(k_z) \) by 7%. Since the effect of the lobes can be removed if they are <50% of the main maximum, the data of Fig. 1 suggest that one-photon 4Pi microscopy should be possible with standard lenses. Unfortunately, the sidelobes observed in practice are generally 10%–15% higher than the theoretical values, which is probably due to residual aberrations introduced by the instrument or by the sample.

To put this expectation to the test, we built a 4Pi module in the type C configuration attached to a beam scanning confocal microscope\(^3\) (Leica Microsystems, Mannheim, Germany). A pair of movable glass wedges placed in each arm of the 4Pi module enabled the achromatic adjustment of constructive interference for both the excitation and the fluorescence wavelengths. In such a 4Pi (type C) system, disparities between the excitation and the fluorescence wavelengths (Stokes shift) can be selected to reduce the sidelobes encountered in \( h_{\text{eff}}(x) \), because the sidelobes of \( h_{\text{exc}}^{\text{4Pi}}(z) \) and \( h_{\text{deg}}^{\text{4Pi}}(z) \) are located at different positions along the \( z \) axis.

First, we measured \( I_z \) by using a monolayer of quantum dots (Qdot 605 streptavidin conjugate, Quantum Dot Corporation) one-photon excited at \( \lambda_{\text{exc}}=488 \) nm and emitting at \( \lambda_{\text{det}}=605 \) nm. The resulting \( z \) response (Fig. 2) agrees well with the theoretical results based on a vectorial calculation.\(^1^2\) However, more important is the fact that the axial sidelobes of ~36% are substantially below the 50% value of the main maximum. Moreover, the axial full width at half-maximum (FWHM) is 95 nm, which signifies a more than fourfold improvement compared with confocal microscopy at the same wavelength.

Fig. 1. (Color online) Axial resolution in 4Pi confocal fluorescence microscopy of type C using one-photon excitation with high-angle immersion lenses: NA 1.20 water, 1.40 oil, and 1.46 oil, as characterized by [(a), left] the axial responses \( I_z(z) \) to an ultrathin plane, and [(a), right] the optical transfer function along the inverse optic axis, \( \text{OTF}(k_z) \). (b) Relative height of the primary axial lobes \( I_{\text{lobes}} \) of \( I_z(z) \), and of \( \text{OTF}(k_z) \) on the semiaerture angle \( \alpha \). An increase of \( \alpha \) by 6° (from 68° to 74°) leads to a 7% decrease of the first-order maximum in the PSF and by the same token to an increase of the OTF at the critical frequency \( k_c \) by 7%. Calculations are for excitation at 488 nm and detection at 605 nm; the pinhole diameter corresponds to half of that of the Airy disk.

Fig. 2. (Color online) Measured \( z \) response to a fluorescent layer of Qdots (excitation, 488 nm; emission, 605 nm). The inset in (b) displays the 4Pi confocal \( xz \) image of the layer. The measured FWHM of the \( z \) response is 95 nm. The corresponding OTF, shown in (b), is contiguous and well above zero; at the critical frequency we have \( \text{OTF}(k_z) > 12\% \).

Fig. 3. (Color online) Side-by-side comparison of focal plane (xy) images of 100 nm diameter yellow–green fluorescent beads recorded by (a) one-photon (\( \lambda_{\text{exc}}=488 \) nm) and two-photon (\( \lambda_{\text{exc}}=820 \) nm) excitation, respectively. The profiles are taken from the same bead. The comparison indicates a slightly improved lateral resolution in the one-photon excitation case.
Additionally, the lateral resolution is slightly improved as compared with the two-photon excitation mode as demonstrated in the measurement shown in Fig. 3. This finding is not surprising, since two-photon excitation of the dye requires an excitation wavelength (here 820 nm) that is almost doubled.

We also recorded microtubules in mammalian cells, stained with the fluorophore DY-485XL (Dyomics GmbH, Jena, Germany) with an emission peak at 560 nm [Figs. 4(e) and 4(f)]. To match the refractive index of the immersion oil, we embedded the cells in 97% 2,2'-Thiodiethanol (TDE). Figure 4 compares xz sections recorded in the standard confocal and in the 4Pi microscopy mode, both with \( \lambda_{\text{exc}} = 488 \) nm. To remove the sidelobe replication, the images were linearly three-point deconvolved; i.e., they were convolved with the inverse function of a simple 1D comb function with one central peak and two lobes. A line profile through one of the microtubules reveals that, in spite of the additional aberrations induced by the biological sample, the side peaks in the 4Pi image are only slightly above 40% of the main peak. The axial FWHM of the profile in the sample was 95 nm [Fig. 4(h)], once again underscoring the fourfold to fivefold improved axial resolution over confocal microscopy. Next we recorded images through microtubules stained with Alexa 488 [Figs. 4(a)–4(c)]. With a fluorescence peak at 519 nm, this dye exhibits a small Stokes shift. Even so, one-photon excitation 4Pi microscopy is viable, as vindicated in Fig. 4(g), thus opening up the prospect for one-photon excitation 4Pi microscopy with dual color labeling.

In conclusion, the advent of objective lenses with ultrahigh semiaperture angles enables 4Pi fluorescence microscopy of biological samples with linear excitation. Cutting implementation costs, this advancement should facilitate a wider application of this superresolving system in biology, especially in light of the fact that our results have been obtained with a commercially available beam scanner. The three-point deconvolution applied in this study just removed the effect of the sidelobes but did not elevate the higher spatial frequencies in the images. The latter can be accomplished by applying a linear\(^{13}\) or a nonlinear\(^{14}\) image deconvolution, in which case the effective axial resolution should be better than 70 nm. Finally, we note that the use of higher aperture lenses is likely to significantly improve the optical performance of the 4PiM as a wide-field 4Pi alternative.\(^{5,6}\)

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References