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4Pi microscopy with negligible sidelobes

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Abstract. The coherent addition of the wavefronts of two opposing high-angle lenses provides an axial (z) resolution improvement by 5–7-fold in far-field fluorescence microscopy. However, all microscopy concepts based on this principle have so far required mathematical deconvolution of the acquired data. This stems from the fact that the decrease of the axial width of the effective point spread function (EPSF) is accompanied by a substantial elevation of the side maxima of the EPSF along the optical axis. Here, we realize an EPSF with negligible lobes and gain axially superresolved images just through the physical phenomena involved. The constructive interference of the added wavefronts can be controlled through the image brightness which greatly simplifies the operation of the system.

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1. Introduction

The three-dimensional (3D) spatial resolution of a conventional far-field optical microscope is well quantified by its 3D-point-spread-function (PSF) describing the diffraction pattern of light around the focal point of the lens \[1\]. For fluorescence imaging, a good measure for the focal plane resolution is the lateral full-width-half-maximum (FWHM) \(\Delta r \approx \lambda/(2n \sin \alpha)\) of the main intensity peak of the PSF. The variables \(n\), \(\alpha\) and \(\lambda\) denote the refractive index, the semiaperture angle of the lens and the wavelength of light, respectively. For widefield fluorescence imaging, the relevant wavelength is that of the fluorescence emission \(\lambda_{fl}\), whereas for a point scanning (non-confocal) arrangement, the PSF is governed by the slightly shorter wavelength \(\lambda_{exc}\) of the excitation light \[1\]. With \(\lambda_{exc} \geq 500\) nm and \(\lambda_{fl} \geq 500\) nm, \(n \approx 1.5\) and \(\alpha < 68^\circ\), the focal plane resolution is limited to \(\Delta r > 180\) nm. A similar equation can be used to assess the FWHM along the optical \((z)\) axis: \[\Delta z \approx \lambda/(n \sin^2 \alpha) > \Delta r,\]

implying an elongation of the main focal spot along the optic axis. According to equation (1), \(\Delta z\) strongly decreases with \(\alpha\), but \(\alpha = 68^\circ\) is the largest angle that has been provided in flat-field corrected lenses so far. According to the scalar equation (1), \(\Delta z \geq 390\) nm, but the vectorial nature of light (which causes deviations from the scalar theory at large focusing angles \(\alpha\)) and residual aberrations render the practical width of the focal spot somewhat larger: \(\Delta r > 200\) nm and \(\Delta z > 600\) nm, meaning that \(\Delta z > 3\Delta r\). In a confocal arrangement, both \(\Delta r\) and \(\Delta z\) can be theoretically reduced by a factor of \(\sqrt{2}\), but the large disparity between the axial and lateral resolution remains \[1\].

The axial elongation of the main diffraction maximum by at least a factor of 3 ultimately stems from a strong asymmetry in the focusing process: the converging wavefront produced by the lens is just a spherical wavefront cap of semiaperture angle \(\alpha \ll 90^\circ\). In contrast, a complete spherical wavefront would be connected with an almost spherical focal spot of diameter \(\sim \lambda/3n\), in which case the axial extent of the main diffraction maximum would be substantially reduced.

2. Principles of 4Pi microscopy

Diffraction is an interference phenomenon and focusing a beam of light is nothing but ensuring constructive interference at a (focal) point \[2\]. Therefore, constructive interference of wavefronts from two opposing high-angle lenses at a common point yields a joint diffraction pattern featuring a main maximum that is narrower along the \(z\)-axis \[3, 4\]. The same consideration applies in reverse: constructive interference of the spherical wavefronts of the fluorescence light at a common point of detection increases the total collection wavefront and thereby reduces the main maximum of the diffraction pattern describing the detection \[3, 4\].

Coherently adding the spherical wavefronts of (two opposing) lenses for excitation and/or fluorescence detection is equivalent to increasing the total aperture of the system, which is the basic idea of 4Pi microscopy, a concept for increasing the axial resolution in (multi)point scanning far-field fluorescence microscopy \[3, 4\]. A schematic diagram of the 4Pi setup is shown in figure 1(a). Laid out in 1990 \[3\], 4Pi microscopy reduces \(\Delta z\) but not \(\Delta r\), since the opposing spherical wavefronts complement each other just along the \(z\)-axis. It is interesting to note that it had been speculated earlier that the focusing of light from all directions with a hypothetical
Figure 1. Enlargement of the aperture angle in 4Pi microscopy provides an axial resolution improvement over confocal fluorescence microscopy that, by not requiring mathematical postprocessing, is entirely based on the physical phenomena employed. (a) Principle of operation: a beam that leaves a (commercially available) confocal laser scanning microscope (not sketched) is divided at a beam splitter (BS) into two counterpropagating partial beams that are focused onto the sample via two opposing lenses (not shown) converting the partial beam’s wavefronts into spherical wavefront caps (blue) of semiaperture angle $\alpha$. In 4Pi microscopy of type A, only the excitation light (blue arrows) is coherently added (at the focus), whereas in type C, the spherical wavefront caps of fluorescence emission (green arrows) is also coherently added at a common point of detection. (b) Objective lenses with semiaperture angle $\alpha = 74^\circ$ enable 4Pi microscopy with an EPSF featuring a main peak with vanishing ($<5\%$) sidelobes. The panels show axial ($xz$) sections of the measured EPSF of a single lens confocal setup (left-hand panel) and a two-photon excitation 4Pi microscope of type C (right-hand panel). Both recordings were performed using the same fluorescent point-like objects and the same type of objective lens. In the confocal case, one-photon excitation was performed at $\lambda_{\text{exc}} = 488$ nm, whereas in the 4Pi case $\lambda_{\text{exc}} = 820$ nm was used for two-photon excitation. The axial FWHM of the EPSF of the 4Pi system is only 105 nm as compared with 370 nm of the confocal reference, as can be inferred from the adjacent profiles along the optical ($z$) axis. Delivering a virtually sidelobe-free super-resolving EPSF, the 4Pi arrangement reaches a major goal in the endeavour of increasing the far-field optical resolution with opposing lenses.

‘4$\pi$ point hologram’ [5] would overcome the diffraction resolution barrier of far-field microscopy per se, by rendering a focal spot with diameter far below $\lambda/2n$. However, this is not possible with freely propagating waves. The improvement in axial resolution by a finite factor as the actual benefit of expanding the full solid angle of the spherical wavefronts had not been recognized. Although it did not break the diffraction barrier, the 3–7-fold axial resolution improvement in 3D imaging brought about by 4Pi microscopy represented an initial step to overcome longstanding barriers in far-field optical resolution [6, 7].

The need for opposing lenses limits 4Pi microscopy and related concepts to imaging fairly transparent objects such as cells or transparent colloidal crystals. As long as the object-induced
aberrations are tolerable, the object thickness is only limited by the free working distance of the high-angle lenses, meaning that object thicknesses up to ~100 µm are possible. The simplest variant of 4Pi microscopy is that of type A where just the excitation light fields are reinforced at the sample. In a 4Pi microscope of type C, the fluorescence wavefronts are also coherently collected by the opposing lenses with their fields adding up constructively at a common point at the detector [4]. A detailed description of the theory of 4Pi microscopy has been given elsewhere [8]–[10]. In brief, the effective point-spread-function (EPSF) of type C is given by

\[
h_{\text{eff}}(\vec{r}) = h_{\text{exc}}(\vec{r}) \cdot h_{\text{det}}(\vec{r}) = \left| \vec{E}_{\text{exc}}(\vec{r}) + \vec{M} \vec{E}_{\text{exc}}(\vec{M} \vec{r}) \right|^{2m} \cdot \left| \vec{E}_{\text{det}}(\vec{r}) + \vec{M} \vec{E}_{\text{det}}(\vec{M} \vec{r}) \right| \otimes p(\vec{r}),
\]

where \( \vec{E}_{\text{exc}} \) and \( \vec{E}_{\text{det}} \) denote the focal fields describing the illumination and the detection by a single lens at \( \lambda_{\text{exc}} \) and \( \lambda_{\text{fl}} \), respectively; \( \vec{r} \) is a spatial coordinate originating at the focal point; \( h_{\text{exc}}(\vec{r}) \) and \( h_{\text{det}}(\vec{r}) \) are referred to as the excitation PSF and the detection PSF, respectively. The transformation matrix \( \vec{M} \) considers the orientation of the counterpropagating light fields. The function \( p(\vec{r}) \) describes the opening of the detector that is conjugate to the focal plane, e.g. the area of a confocal ‘pinhole’ [8]. If no pinhole is used, \( p(\vec{r}) = 1 \). The parameter \( m = 1, 2, \ldots \) gives the number of photons involved in the excitation process, e.g. \( m = 2 \) for two-photon excitation.

A challenge in realizing concepts relying on the coherent use of opposing lenses is that the maximum semiaperture angle provided by standard high-angle lenses (\( \alpha = 68^\circ \)) is substantially smaller than the 90° required to form a half-sphere [8]. Because of this, the EPSF not only comprises an axially narrowed main maximum, but also higher-order lobes above and below the focal plane. For \( \alpha = 68^\circ \), the first-order lobes practically amount to ~50% of the main maximum and hence are too prominent to be neglected. The reason is that these lobes contribute to the image formation by generating periodic artifacts in the image. An investigation of the Fourier transform of the diffraction intensity pattern reveals that in order to be tractable, the effective signal contribution of an individual lobe to the image has to be lower than ~50% of the main maximum [8]. This condition applies not only to 4Pi microscopy, but also to the related scheme of IM [11]–[13].

Although non-confocal versions have been reported [14, 15], 4Pi microscopy has mostly been implemented with confocal detection, because by suppressing the fluorescence signal from outside the focal plane, confocality dampens the contribution from the lobes. The type C implementation also reduces the effective sidelobe height due to the disparity between \( \lambda_{\text{exc}} \) and \( \lambda_{\text{fl}} \). This disparity causes the sidelobes of \( h_{\text{exc}}(\vec{r}) \) and \( h_{\text{det}}(\vec{r}) \) to emerge at different points along the \( z \)-axis, while the main maxima still peak at the common focal point at \( \vec{r} = 0 \). Hence, due to \( \lambda_{\text{fl}} \neq \lambda_{\text{exc}} \), the fluorescence from the sidelobes is less efficiently collected than that of the main peak.

Another powerful mechanism of lobe suppression is two- or three-photon fluorescence excitation \( (m = 2 \text{ and } 3) \), because the quadratic or even cubic dependence of the fluorescence signal from the local intensity weakens the contributions from the lobes [16]. Applied in a 4Pi microscope of type C, multiphoton excitation is particularly effective because in this case \( \lambda_{\text{exc}} \) is much longer (750–1100 nm) than \( \lambda_{\text{fl}} \), which remains in the visible range (450–700 nm). Again, the discrepancy in wavelengths leads to lobes that fall on different coordinates along the \( z \)-axis, meaning that the signal from the lobes is suppressed by low values of detection [15, 16]. Thus, the combination of multiphoton excitation, confocal detection and wavelength disparity yields the lowest lobes. For example, with typical \( \lambda_{\text{exc}} = 800 \text{ nm} \) and \( \lambda_{\text{fl}} = 550 \text{ nm} \), a two-photon

\[ \text{http://www.njp.org/} \]
excitation 4Pi confocal fluorescence microscope of type C using lenses of $\alpha = 68^\circ$ should display axial lobes of $\sim 9\%$. However, due to aberrations, lobes $< 10\%$ have not been obtained in practice. Rather, sidelobes of 20–30% were typically achieved in measurements. Thus, the direct interpretation of the acquired data is precluded by lobes $> 10\%$ of the main peak.

To deal with the artefacts induced by the lobes in the image, the raw image data have been processed mathematically. A minimal algorithm is a so-called point-deconvolution, a simple comb-like filter removing just the replication introduced by the lobes [17]. Another option is a linear deconvolution with the whole EPSF [18]. Deconvolution with the whole EPSF is mandatory in the 4IM where the lobe height is at the largest acceptable value of 50% [12]. Applying deconvolution is always challenged by noise, especially if the lobes are $> 20\%$ or if the signal is weak. Therefore, a major goal in this endeavour is the development of an optical scheme featuring a single, nearly spherical focal peak.

In the last few years, significant progress has been reported towards this goal. For example, it has been shown that the implementation of ring-shaped obstructions (dark rings) in the entrance pupil of high-angle lenses yields lobes of 7–14% in a two-photon excitation 4Pi microscope [19, 20]. However, the obstructions discard a large fraction of both the excitation and the fluorescent light and lead to a lobe-induced background in the image. Another option is the suppression of the lobe fluorescence by stimulated emission [21], but this measure requires an additional beam of light.

### 3. Results and discussion

We now describe the generation of a single, sharply reduced focal spot by realizing two-photon excitation 4Pi microscopy of type C with novel oil-immersion lenses featuring an enlarged semiaperture angle of $\alpha = 74^\circ$ [22, 23]. The result is a double-lens microscope featuring the largest clear aperture currently possible, and which improves the axial resolution all-optically.

Figure 1(a) sketches the basic optical scheme of a 4Pi microscope. While the lenses are not sketched in this drawing, the two counterpropagating spherical wavefronts formed by these lenses (either for illumination or detection) are indicated, along with the increase in $\alpha$ from 68° to 74°. Figure 1(b) displays $xz$-sections of the measured EPSF of the confocal and of the corresponding 4Pi system for constructive interference. Both EPSFs were recorded by $xz$-scanning a 100 nm diameter fluorescent bead (yellow-green fluorospheres, Invitrogen, Eugene, OR, USA) in a plane containing the optical (z) axis (figure 1(c)). The absorption of the fluorophore peaked at 505 nm, whereas its emission peaked at $\lambda_{fl} = 515$ nm. Performed in the one-photon excitation mode at $\lambda_{exc} = 488$ nm, the recording of the confocal EPSF features an FWHM of $\Delta z = 370$ nm, which compares well with the $\Delta z = 320$ nm derived from numerical calculations. Note that these values are improved over the $\Delta z > 400$ nm typically reported in confocal systems at this wavelength because of the increase in $\alpha$ brought about by the novel lens. Featuring an axial FWHM of 105 nm, the corresponding EPSF of the two-photon 4Pi setup is still 3.5 times narrower than the confocal reference. Moreover, the sidelobes are $< 5\%$, which is beneath the noise level in most biological applications. Thus, we obtain a nearly sidelobe-free EPSF rendering mathematical deconvolution obsolete.

The formation of a single peak in the 4Pi system is due to the synergy between the expansion of $\alpha$, confocality, and two-photon excitation ($m = 2$) [24, 25]. While two-photon excitation reduces the FWHM of the main maximum of the EPSF by $\sqrt{2}$, this small improvement is not able to make up for the enlargement of the FWHM resulting from the longer
wavelength $\lambda_{\text{exc}} = 820\,\text{nm}$ (as compared to the $\lambda_{\text{exc}} = 488\,\text{nm}$ used for one-photon excitation). Therefore, the only benefit of $m = 2$ is the suppression of the lobes by the weaker excitation at the spot periphery and the disparity between the excitation and emission wavelengths at $\lambda_{\text{eff}} = 515\,\text{nm}$.

The increase in aperture angle renders the EPSF more vulnerable to aberrations induced by the object. A major source of spherical aberration is a potential mismatch between the refractive index of the embedding medium and the immersion oil of the objective lens whose refractive index at the sodium D-line is given by $n_{23}^{D} = 1.515$ at $23^\circ\text{C}$. The refractive index-induced aberrations spoil the axial resolution in a confocal system [26], whereas in 4Pi microscopy they increase the sidelobes and cause the phase difference between the counterpropagating wavefronts to vary with the penetration depth of the joint focal point in the sample [27]. While non-biological samples, such as colloidal nanoparticles, can be mounted in immersion oil, even fixed biological samples have to be embedded in a chemically less aggressive medium featuring a refractive index that matches the average index of the sample. We have identified TDE (2, 2'-thiodiethanol; Sigma–Aldrich) as a suitable mounting medium that is arbitrarily miscible with water and even acts as an antioxidant [28]. To match the refractive index to $n = 1.515$, we added 3% phosphate buffer to yield 97% TDE; its precise value was adjusted using a refractometer. We used coverslips of 160 $\mu\text{m}$ thickness, which we found to give the best tolerance for the setting of the in-built aberration correction collar of the lenses. The settings of the correction collars were optimized for a wavelength of 647 nm by maximizing the reflection of the focused light from a mirror.

For two-photon excitation at $\lambda_{\text{exc}} = 820\,\text{nm}$, the beam of a mode-locked Ti:sapphire laser (MaiTai, Spectra Physics, Mountain View, CA, USA) was coupled to a standard beam-scanning confocal microscope (TCS SP2, Leica Microsystems CMS, Germany) via its infrared port. The microscope turret of the scanner was replaced by a home-built 4Pi unit, which is described in detail elsewhere [15]. In brief, the beam emerging from the confocal scanner is divided at a beam splitter and subsequently focused onto the sample by two opposing objective lenses of $\alpha = 74^\circ$ (PL APO 100 × 1.46 numerical aperture (NA)) oil immersion by Leica Microsystems CMS). The emitted fluorescence light is collected by both objectives and merged at the beam splitter. The fluorescence light was detected with a photon-counting avalanche photodiode. The adjustment of the two objectives was actively maintained using piezoelectric actuators operating in a closed loop.

To add the fluorescence light fields coherently, the optical path difference and the dispersion were adjusted in the 4Pi setup. Two sliding glass wedges allowed us to establish a constant phase over a wavelength regime broader than the spectral bandwidth of the fluorescence light [15]. Furthermore, the sliding wedges enabled us to define the optical path difference between any two wavelengths in use. For example, it was possible to preset constructive interference both of the excitation light ($\lambda_{\text{exc}} = 820\,\text{nm}$) at the sample and of the collected light ($\lambda_{\text{det}} = 530\,\text{nm}$) at the detector, a setting that we refer to as ‘c/c’. Likewise, it was possible to install constructive interference for excitation and destructive interference for fluorescence at the confocal detector, which we name ‘c/d’.

To demonstrate the control of the phase difference and, hence, of the resulting EPSF, we investigated a layer of fluorescent quantum dots (Qdot 525 Streptavidin Conjugate, Quantum Dot Corporation) (figures 2(a) and (b)) and compared the experimental results with calculations based on the vectorial focusing theory of Richards and Wolf [29, 30]. For all calculations, a pinhole diameter of half the Airy disk diameter was assumed. The $z$-response [1], defined as the
Figure 2. Experimental and theoretical z-responses of a two-photon excitation 4Pi microscope of type C measured with a layer of quantum dots ($\lambda_{\text{exc}} = 820$ nm and $\lambda_{\text{fl}} = 525$ nm) for (a) in-phase (c/c) and (b) dephased (c/d) interferences of the wavefronts of the opposing 1.46 NA lenses. The peak of the c/d-EPSF is reduced by 64% as compared with that of the c/c case; the integral signal is decreased by 20%. In an imaging application, microtubules stained with Alexa 488 were measured at $\lambda_{\text{exc}} = 780$ nm at the same phase differences (c)–(f). The xy-images in the c/c (c) and the c/d-case (d) confirm the strong decrease in signal intensity. The $xz$-slices shown in (e) and (f) feature low axial lobes and modulate strongly in intensity with changing phase difference. Note that the drastic change in total image brightness enables the adjustment of constructive interference of the complementing wavefront pairs.

response to an infinitely extended plane scanned along the optic axis, quantifies the ability of a 3D imaging system to discern axially stacked planes:

$$I_z (z) = \iiint h_{\text{eff}} (\vec{r}) \, dx \, dy.$$  

We performed measurements of the z-response at extreme phase settings, namely at constructive interference of excitation and detection (c/c), as well as at constructive excitation and destructive detection (c/d). The phase differences were attained by changing the optical path length in one of the 4Pi arms. The c/c setting features the brightest response (figure 2(c)) of all possible responses; the c/d response is much dimmer (figure 2(d)). In both cases, we obtained good agreement between the measurement and the theory. The marginal differences between theory and measurement are due to experimental imperfections, such as residual aberrations, unequal intensities in the two 4Pi arms, and polarization-dependent phase changes at the beam
splitter. In the c/c case, the sidelobes of the z-response are well below 10%. Representing the integral of the EPSF in the xy-plane, the z-response is an upper bound for the lobe height in the sample.

More important is the fact that the (c/c)-z-response is considerably stronger than its (c/d) counterpart both in peak and in total intensity. In the (c/d) case, the peak amounts only to ∼36% of its (c/c) counterpart. Also, in the (c/d) case the total signal \( \int I_z(z) \, dz \) is reduced to 80% of that for (c/c). These notable differences in brightness represent a further important advancement, because so far, 4Pi microscopy and related methods have required the concomitant imaging of point-like or planar structures to adjust the relative phase. Figure 2 shows that constructive interference can now be adjusted through the image brightness, another long-sought goal in the development of this system [31]. Note that if the EPSF is just ‘modulated’, as is the case in a standing wave microscope, the difference in peak height encountered in practice is masked by noise. By contrast, the production of a solitary peak renders an EPSF whose dephasing can be readily defined by its relative decrease in brightness, in analogy to the Strehl ratio in single lens imaging.

To demonstrate this advancement, we measured microtubules in a fixed mammalian (PtK-2) cell stained with an organic dye (Alexa 488 goat anti-rabbit IgG, by Invitrogen). Figures 2(c)–(f) exhibits that the (c/c)-phase setting yields a significantly (∼3 times) brighter image as compared with the (c/d) case. The \( xz \)-slices through the data sets (figures 2(e) and (f)) demonstrate that the EPSFs measured in the biological sample agree well with the results obtained on the Qdot sample.

This finding is confirmed in a further 3D-imaging experiment on microtubules in neuroblastoma cells stained with Alexa 488 (figures 3(a)–(c)). Figure 3(b) directly compares the 4Pi raw data with the same data after a linear deconvolution with the EPSF. The difference between the images is negligible since the sidelobe effects in the raw data are already in the range of the background. A profile through an inherently line-shaped microtubule shows that a single sharp central spot with an axial FWHM of about 110 nm is formed (figure 3(c)), indicating an axial resolution increase by a factor of ∼3.5 compared with the confocal measurement. The confocal data were recorded by blocking one of the arms of the 4Pi microscope at \( \lambda_{\text{exc}} = 488 \, \text{nm} \).

The low sidelobes also indicate the good match between the average refractive indices of the sample and the embedding medium TDE. Another indication of the match is the negligible variation in phase difference when scanning the focal spot through the sample. For example, we measured microtubules in PtK-2 cells stained with Alexa 488 that were located close to the nucleus (figure 3(d)). The line profile through the nucleus (indicated in blue) shows that the phase shift due to refractive index mismatch is negligible (figure 3(e)). Both the microtubules above and below the nucleus indicate constructive (c/c) interference between both counterpropagating wavefront pairs.

Figures 3(f) and (g) display two 3D surface rendered images of mitochondria (figure 3(f)) and microtubules (figure 3(g)) in PtK-2 cells stained with Rhodamine (Carboxyl Rhodamine Sheep Anti-Mouse IgG) and Alexa 488, respectively. The raw data obtained in the 4Pi recording (at \( \lambda_{\text{exc}} = 820 \, \text{nm} \)) were directly visualized with the image rendering software Amira (version 3.1.1, TGS Inc., San Diego, CA, USA) without prior deconvolution. Above and below each structure, no artefacts caused by sidelobes can be observed. The results confirm that no deconvolution is necessary to interpret the data obtained by two-photon 4Pi microscopy of type C using these high-NA lenses.
Figure 3. Axial superresolution imaging of the microtubular network in neuroblastoma cells stained with Alexa 488 (a)–(c). An xy-projection (a) marks the position of the xz-section shown in (b). A one-photon excitation confocal xz-image ($\lambda_{\text{exc}} = 488\,\text{nm}$) is compared with the raw data of the two-photon excitation 4Pi type C ($\lambda_{\text{exc}} = 820\,\text{nm}$) recording (centre panel in (b)), and with the same 4Pi data set after a linear deconvolution. The deconvolution shows only marginal improvements in lobe suppression, since the lobe-induced effects in the 4Pi raw data are weak, as confirmed by the z-profile (c) derived by integrating in the boxed area in (b). An xz-section (d) through the nucleus (indicated in blue) of an Alexa 488-stained microtubular network in a mammalian (PtK-2) cell demonstrates that the embedding medium TDE allows precise matching of the refractive index to that of the immersion system: no significant phase shift due to the focusing through the cell nucleus was found (e). Raw 4Pi microscopy 3D data of mitochondria (f) stained with Rhodamine and microtubules (g) stained with Alexa 488 in PtK-2 cells visualized by 3D-surface rendering.

Nonetheless, the resolution can be further increased by applying image processing algorithms, such as a one-step linear deconvolution with the EPSF (Wiener filtering) or a nonlinear iterative Richardson–Lucy image restoration [32, 33]. The raw confocal and 4Pi data shown in the upper row of figure 4(a) were deconvolved by applying these two algorithms. In

Figure 4. Confocal (left column) and 4Pi (right column) xz-images through the microtubular network in a PtK-2 cell stained with Rhodamine. The raw data (upper row), and the result after a linear deconvolution using a Wiener filter (middle) and Richardson–Lucy restoration (lower panel) are compared. Line profiles through the data sets are shown in (b) for the confocal ($\lambda_{\text{exc}} = 568$ nm) and in (c) for the 4Pi measurement ($\lambda_{\text{exc}} = 820$ nm). While restoration enhances the resolution further, the substantial increase in axial resolution brought about by the 4Pi recording is already evident in the raw data of the upper right panel.

both data sets the resolution is increased, as can be observed by comparing the corresponding xz-images (figure 4(a)) or the pertinent data line profiles for the confocal (figure 4(b)) and for the 4Pi measurement (figure 4(c)). In the raw data as well as in the mathematically enhanced data, the 4Pi measurement yields superior axial resolution compared with the confocal measurement. Importantly, the raw 4Pi data already reveal most of the details in the sample, confirming that the majority of the resolution improvement is due to the physics of 4Pi imaging, i.e. to the increase in aperture solid angle.

Figure 5. The amplitude of the theoretical OTFs of a confocal and a 4Pi microscope for NA = 1.40, 1.46 and 1.51 (oil immersion) corresponding to $\alpha = 68^\circ$, 74$^\circ$ and 85$^\circ$, respectively, are displayed and compared with the amplitude of the OTF derived from the 4Pi raw data shown in figure 4(a). Note the excellent agreement between the theoretical 4Pi OTF and its experimental counterpart for the NA = 1.46 lenses used. Comparison with the confocal OTFs shows a 3–4-fold enlargement of the axial bandwidth, with the OTF displaying only a small dent at $k_z \approx 12$. Increasing the semiaperture angle further to 85$^\circ$ would only lead to marginal improvements of the OTF.

Taking advantage of the presence of point-like objects in the raw 4Pi data, we derived the experimental optical transfer function (OTF), i.e. the spatial Fourier transform of the EPSF, for the NA = 1.46 ($\alpha = 74^\circ$) lenses used and compared them with the theoretical OTFs for the same NA, calculated using the theory of Richards and Wolf. Figure 5 exhibits excellent agreement between the measured and the calculated OTF amplitude. The frequency bandwidth transmitted in the 4Pi arrangement is larger by a factor of $\sim 4$ compared with the confocal case, underscoring the improved axial resolution of the 4Pi microscope. Most importantly, the 4Pi OTFs are fully contiguous and, apart from a small dip, they resemble a Gaussian function, in accordance with the fact that the almost solitary main peak can loosely be approximated by a Gaussian function, as well.

Therefore, the question arises as to whether a further increase in $\alpha$ would lead to further OTF improvements. We calculated the 4Pi OTF amplitude also for $\alpha = 68^\circ$ and 85$^\circ$, corresponding to oil immersion NAs of 1.40 and 1.51, respectively. The comparison with the OTF observed in our experiments reveals that the expected improvement at $\alpha$ approaching 90$^\circ$ is rather marginal, namely the removal of the small dip in the OTF. The only noteworthy benefit of a larger $\alpha$ would be a larger difference in peak intensity between the (c/c) and (c/d) EPSFs, i.e. larger than what is obtained in figures 2(c) and (d). While such an increase in contrast eases the adjustment of constructive interference, it comes at the expense of an increased susceptibility to aberrations. So far the solitary peak has been demonstrated with fixed cells only, which is due to the fact that no water immersion lenses are available with improved semiaperture angle yet. However, the results herein show that improving the angle for water immersion lenses should greatly improve the axial resolution capability of 4Pi systems also for live cell imaging.
4. Conclusion

Exploiting the common nature of diffraction and interference, 4Pi microscopy improves the 3D resolution in far-field fluorescence microscopy by coherently adding the spherical wavefronts of two opposing lenses. Two-photon excitation and fluorescence collection with constructively interfering spherical wavefront caps of semiaperture angle \( \alpha > 74^\circ \) yield an EPSF with a solitary main maximum that is 3–4 times narrower along the z-axis than in a standard confocal fluorescence microscope. This resolution improvement is also reflected in the nearly 4-fold enlarged bandwidth of the OTF of the system.

The all-optical creation of a solitary focal peak has been a major goal in the quest of improving the spatial resolution with opposing lenses of limited semiaperture angle \( \ll 90^\circ \), because in this way many of the remaining issues connected with this approach are alleviated or solved. An obvious advantage of a solitary main maximum is that the images are directly formed while being recorded. Since mathematical postprocessing is not required, the quantitative exploitation of the data is greatly simplified and less prone to noise artefacts. Last but not least, the system is able to self-indicate the correct superposition of the spherical wavefronts. Dephasing is effectively an aberration of the ‘4Pi’ focusing system, much the same way as an ordinary wavefront dephasing represents an aberration for a single lens. As a result, in-phase images are bright, whereas the dephased images are significantly darker.

Finally, we note that the improvement reported in this paper is, to a large extent, due to a moderate increase of 6° in the aperture angle (from 68° to 74°) in conjunction with a thorough refractive index match of the sample with that of the immersion system. Thus, our results underscore once more that the enlargement of the aperture angle and the concomitantly expanded coverage of spatial frequencies and not the mere creation of interference peaks is the essential physical element in the resolution improvement with opposing lenses.

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References
