4Pi microscopy of type A with 1-photon excitation in biological fluorescence imaging

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Abstract: We demonstrate that oil immersion lenses with a semi-aperture angle \( \geq 74^\circ \) enable 4Pi confocal fluorescence microscopy of type A with 1-photon excitation. The axial sidelobes amount to \(< 50 \%\) of the main diffraction maximum, implying that lobe induced artifacts can be removed from the image data. The advancement reported herein enables a relative inexpensive implementation of 4Pi microscopy, providing axially superresolved 3D-imaging in transparent samples. As an example, we show dual-color 4Pi images of double stained Golgi stacks in a mammalian cell with 110 nm axial resolution. The resolution can be further enhanced to values slightly below 100 nm by image deconvolution.

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References and links

1. Introduction

4Pi [1,2] and 15M [3,4] fluorescence microscopy deliver axial super-resolution by coherently adding the spherical wavefront caps of two opposing high angle lenses. Constructive interference of the two wavefronts produces a main diffraction maximum of their effective point-spread-function (PSF) that is 3-7 times narrower than that of a single lens, albeit at the expense of elevated lobes along the optic axis (z). The height of the lobes sharply decreases with increasing semiaperture angle \( \alpha \), making the use of high angle lenses mandatory for these techniques. A challenge in 4Pi and 15M imaging is that the narrower main maximum can only be exploited if the enlarged primary axial lobes of the PSF are <50% of the main maximum; otherwise, the image contains ambiguous data. This condition is more easily attained in scanning 4Pi microscopy as compared to widefield 15M, because single or multi-point scanning offers the option to implement a confocal pinhole in the detection path to discriminate the fluorescence from further up or down the optic axis. Thus, according to the theory, an immersion lens with \( \alpha = 68^\circ \) should yield a PSF with lobes < 50%.

Oil immersion lenses of 1.4 numerical aperture (NA) and glycerole lenses of NA = 1.35 are such lenses. Calculations show that, with these lenses, also the most basic version of 4Pi microscopy should be viable, which is the 4Pi confocal microscope of type A using 1-photon fluorescence excitation [1]. Type A means that only the illumination wavefronts are added at the common focal point of the opposing lenses; the fluorescence is collected incoherently. This is in contrast to 4Pi microscopy of type C [1] and to 15M [3] where the emitted spherical wavefronts of fluorescence need to be coherently collected, as well. All experimental efforts to implement a simple 1-photon excitation 4Pi microscope of type A [2] failed in the past, because confocalization alone was insufficient to suppress the lobes below 50%. This was the case for all aplastic lenses featuring the largest hitherto available semiaperture angle \( \alpha = 68^\circ \), irrespective whether they were oil or glycerole immersion lenses. Therefore, the implementation of further lobe suppression mechanisms was required, such as fluorescence excitation by 2-photon absorption [5] and/or coherent fluorescence collection. In fact, virtually all reported cell biology applications of 4Pi microscopy relied on 2-photon excitation [6]. Although very effective, 2-photon fluorescence 4Pi microscopy requires sophisticated lasers and, with a few exceptions [7], suffers from a lower fluorescence yield.

The progress reported herein has become possible by the recent advent of aplanically corrected oil immersion lenses (HCX PL APO CS 100/1.46 Oil, Leica Microsystems, Germany) featuring a larger angle: \( \alpha = 74^\circ \). We now show that, in combination with the new mounting medium thiodiethanol [8], the expansion of \( \alpha \) from the standard \( 68^\circ \) to \( 74^\circ \) reduces the axial lobes of a 1-photon excitation 4Pi confocal fluorescence microscope of type A below the 50% threshold. This progress in lens design enables a comparatively simple 4Pi imaging modality with brighter images as compared to the standard 2-photon 4Pi microscopy mode. Moreover, it can be easily implemented in standard beam scanning 4Pi microscopes, including in those that are commercially available. Furthermore, we show this far-field microscopy to provide dual-color axially super resolved images of biological cell organelles.

2. Theory

The effective PSF of a confocalized 4Pi fluorescence microscope of type A using 1-photon excitation is given by

\[
h_{\text{eff}}(r) = h_{\text{exc}}^{4\Pi} \cdot h_{\text{det}}^{4\Pi} = \left| E_{\text{exc}}(r) + \hat{M} E_{\text{exc}}(\hat{M} r) \right|^2 \cdot \left| E_{\text{det}}(r) \right|^2 \otimes p(r) \tag{1}
\]

where \( E_{\text{exc}} \) and \( E_{\text{det}} \) are the focal fields for excitation and fluorescence detection, respectively, \( \hat{M} \) is a transformation matrix accounting for the counter-propagation of the beams and \( p(r) \) denotes the pinhole function [9]. The line profile along the optic axis is given by
$h_{\text{eff}}(0,0,z) = h_{\text{eff}}(z)$ and the $z$-response to an infinitely extended plane is $I_z(z) = \int \int \int dx dy h_{\text{eff}}(r)$.

The calculated dependence of the lobe height of $h_{\text{eff}}(z)$ and the $z$-response $I_z(z)$ on $\alpha$ is shown in Fig. 1(a). Using $\alpha = 74^\circ$ instead of $68^\circ$ reduces the lobe height of the $z$-response by 7% (in absolute percentage). Further increase of the semi-aperture angle would decrease the lobes further and therefore render the presented method even more robust. Figure 1(b) shows the behavior of the axial and lateral full-width-half maximum (FWHM) of the main maximum of the PSF of the 4Pi microscope of type A with 1-photon excitation at 488 nm and central emission wavelength at 515 nm. With increasing $\alpha$, the axial FWHM increases slightly because larger spherical wavefront caps imply more wave components traveling at a greater angle to the optic axis. The lateral FWHM decreases with $\alpha$, as expected. However, increasing $\alpha$ also increases the demands on the optical properties of the sample. An effective solution to the growing refractive index problem is TDE (2,2'-Thidodiethanol, Sigma-Aldrich, St. Louis, MO, USA), a water-miscible mounting medium allowing the tuning of the refractive index of the embedding medium from 1.33 up to 1.52 [8]. To avoid spherical aberration when applying the NA=1.46 oil immersion lenses, we adjusted the refractive index of the sample to 1.518, i.e. to that of the glass-oil system. To minimize bleaching, we added Cysteamine (Sigma-Aldrich, St. Louis, MO, USA) and Dabco (1,4-Diazabicyclo[2.2.2]octane, Alfa Aesar GmbH, Karlsruhe, Germany).

Figure 2(a) shows the PSF calculated with a high-angle focusing vectorial theory [10], at the above wavelength conditions. The confocal pinhole diameter was set to 0.5 Airy units. Figures 2(b) – 2(d) show the PSFs after convolution with a 100 nm diameter sphere, a 100 nm thick rod extending in the $y$-direction, and a 100 nm thick $xy$-plane, respectively.
The line profiles along the optic axis (z) reveal that the sidelobe issue is more severe for laterally extended objects. The sidelobes of the sphere and the rod images are both ~30% which is well below the 50% value. In practical biological imaging, the sidelobes are by ~10% larger than in theory, implying that while the imaging of point-like and rod-like features is readily viable in 1-photon 4Pi microscopy of type A, the imaging of extended layers has to be exerted with care. Therefore, the successful exploitation of this imaging mode depends on the fulfillment of the optical conditions to be observed: refractive index matching and thorough setup alignment. If these conditions are met, 1-photon 4Pi microscopy of type A renders axially superresolved images also for axially layered structures.

Theory also shows that the sidelobe height hardly varies with the excitation wavelength applied. From Fig. 2, it can also be seen that the first minima are filled for axially extended objects, allowing for assessment of the thickness of the measured object [9, 11].

3. Experiments and applications

We employed a home-built 4Pi module that was attached to a standard beam scanning confocal microscope (Leica Microsystems, Mannheim, Germany) featuring two opposing NA=1.46 oil immersion lenses. Each arm of the 4Pi module featured movable glass wedges which are normally used to compensate dispersion in a type C adjustment [12]. In order to avoid type C conditions, we detuned the wedges such that the optical path difference in both 4Pi arms was larger than the coherence length of the fluorescence light. Thus, the fluorescence light was collected incoherently.

First, we performed experiments on red fluorescent beads (λ_em=605 nm) that were fixed on a coverslip of 150 μm thickness and subsequently embedded in 97% TDE [8]. Figure 3 shows the line profile measured on a 100 nm diameter bead excited at λ_exc=568 nm. The measured sidelobes of 35% are in good agreement with the theoretical prediction. Both the theoretically expected and the measured FWHM of the main maximum is 140 nm. The calculated FWHM of a point-like object at the same excitation wavelength is 126 nm.
The viability of this 4Pi microscopy mode is further exemplified in Fig. 4 where we display an axial (xz) image section through the Golgi complex of a fixed mammalian Vero cell. Specially, we imaged the endogenous protein GM 130 as a marker for the cis-Golgi compartment immunolabelled with the organic fluorophore Cy3 (Dianova, Hamburg, Germany) excited at $\lambda_{\text{exc}}=568$. Figure 4 also displays the confocal data and the 4Pi data after sidelobe removal by a linear 3-point deconvolution. The latter means that the images are convoluted along the z-axis with the inverse of a 1D comb function consisting of a central spike and two or four additional spikes of alternate sign [13].

Mathematically, this is a genuine deconvolution that only removes the sidelobes and does not enhance the resolution. Unless a background is subtracted, it is fully reversible. The raw 4Pi data in Fig. 4 exhibits sidelobes of ~50% that can be removed satisfactorily, resulting in a 4Pi image that is much more faithful than the corresponding confocal measurement. The xz-sections feature 409 x 155 pixels of size 27 x 16 nm. The signal-to-noise ratio of the raw data was 7:1.
Figure 5 displays 3D surface rendered representations of confocal and 4Pi confocal stacks of xz-images of the distribution of the *cis*-Golgi protein GM130 immunostained with carboxyl-Rhodamine 6G. Emitting orange fluorescence, this fluorophore was also excited at $\lambda_{\text{exc}}=568$ nm. The complete data stack consists of 247 xz-images of 16.6$\mu$m x 5.0$\mu$m in size. To avoid temperature drifts during 3D-image acquisition, we used an automatic alignment of the 4Pi setup. The 4Pi recording shows a much more detailed structure than the corresponding confocal data.

Imaging the distribution of two distinct and differently labeled proteins is of enormous importance in cell biology. This stems from the fact that in most cases, it is not so much the absolute distribution of a particular protein that is of interest, but its spatial relationship to another protein in the cell. Hence, we investigated the capability of this 4Pi imaging mode to image two fluorescent dyes at the same time or consecutively.

To this end, *Vero* cells transiently transfected with a Sulfotransferase-GFP hybrid localizing in the *trans*-Golgi were subsequently immunostained with Alexa546 (Invitrogen, Karlsruhe, Germany) via an anti-GFP antibody (Biozol, Eching, Germany). The second color targeted the *cis*-Golgi protein GM130; it was stained with the red emitting fluorophore Alexa647 (Invitrogen, Karlsruhe, Germany). Excitation was performed at 568 nm and at 647 nm for Alexa546 and Alexa647, respectively. A dichroic mirror (Z690SPRDC, Chroma Technology Corp, Rockingham, USA) separated the two emission channels. The fluorescence was detected with two counting avalanche photodiodes.

The excitation wavelength was swapped after each frame, so that the two emission colors were measured sequentially. The emission crosstalk between the detection channels was negligible. Also, we did not observe a noteworthy axial chromatic aberration. Figure 6 shows the image data for Alexa546 (green) and Alexa647 (red) for each color channel: 4Pi raw (a, d), confocal (b, e), and 4Pi after removal of the sidelobes by 3-point deconvolution (c, f). The 3-point deconvolution was successful due to the fact that the average lobe height was slightly below 50%. In the case of the 4Pi recording, the overlay of the two color channels gives a detailed image, making *cis*- and *trans*- localized Golgi proteins clearly distinguishable. Additionally we performed a Richardson-Lucy algorithm [14] which is able to restore frequencies that are more weakly transmitted [Fig. 6 (i), 6(j)].

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The restoration leads in both cases to an enhanced resolution, but still in the 4Pi measurement the resolution is higher than in the confocal counterpart. The reason for choosing this pair of dyes was the fact that, for the utilized 1.46 NA lenses, the longitudinal (i.e. axial) chromatic aberration was negligible in the yellow red wavelength range. Shorter excitation wavelengths are expected to yield a slightly better resolution. Therefore, we investigated the effect of longitudinal chromatic aberrations on the 4Pi imaging mode of type A. We double-stained the Golgi apparatus in the same way as previously, but this time we utilized Bodipy FL (Invitrogen, Karlsruhe, Germany) for marking the \textit{trans}-Golgi ($\lambda_{\text{exc}}$=488 nm) and Alexa546 for highlighting the \textit{cis}-Golgi apparatus ($\lambda_{\text{exc}}$=568 nm). The images were recorded sequentially, meaning that after each frame, the excitation wavelength was swapped and the focus of one of the lenses was readjusted toward an optimal PSF of the 4Pi system. Additionally, the phase was slightly shifted to adjust constructive interference. The correction of the longitudinal chromatic aberration, by displacement of a single lens, leads to a positioning error which requires manual readjustment by subtraction of half of the initial displacement.

Figure 7 compares the confocal and 4Pi imaging results. The sidelobes for excitation at 488 nm are more pronounced due to larger residual aberrations at the shorter wavelength. Additionally, the finite thickness of the Golgi stacks causes a weaker modulation at the shorter wavelength.
4. Discussion and Conclusion

Oil immersion lenses of semiaperture angle $\alpha = 74^\circ$ (NA=1.46) enable a simple variant of 4Pi microscopy: 1-photon excitation of type A. It is simple because it relies just on the coherent addition of the illumination wavefronts and because it requires inexpensive laser sources. Compared to the hitherto employed 2-photon excitation, 1-photon fluorescence excitation generally provides brighter images and also a slightly better axial resolution. The latter stems from the fact that the utilized wavelengths are shorter for the 1-photon excitation: 450-650 nm as compared to the 750-1100 nm wavelength for its nonlinear counterpart. Calculations predict that, depending on the excitation wavelength, 1-photon excitation 4Pi microscopy of type A provides an axial resolution of 100-130 nm in 3D-microscopy. Our experiments confirm the theoretical prediction for the wavelengths investigated. The wavelength of the fluorescence emission is of much lesser importance, since the fluorescence light is collected incoherently. The Stokes shift influences the confocal suppression of the axial lobes, but plays a small role in the resolution performance of the system. The resolution can be further augmented to 80-100 nm by applying (non-)linear mathematical post processing using the measured PSF of the system [13, 15].

The novel large angle lenses can be readily incorporated into existing 4Pi units, including those that are commercially available. To fulfill the <50 % lobe condition, one has to observe that the setup is adjusted and that the embedding medium is well matched to the rest of the optical system. The novel mounting medium thiodiethanol has proven adequate for tuning the refractive index of the sample in fixed cells. Therefore we expect our findings to facilitate further applications of 4Pi microscopy in biology.

The applicability of 1-photon 4Pi microscopy of type A is also confirmed by the fact that dual color imaging is readily possible, as has been demonstrated here both for yellow-red as well as for blue-yellow emitting dye pairs. Additional alignments were needed in the blue-yellow range to compensate for residual longitudinal chromatic aberrations - at least for the lens duo used herein. This problem may be alleviated in the future either by improving the lenses or by implementing corrective optical elements. In any case, the straightforward
practicability of dual-color biological imaging in the yellow-red regime should help in elucidating many structural details of the 3D-distributions of proteins in cells.

Our work has shown that the key to the viability of 1-photon excitation 4Pi fluorescence microscopy of type A is the larger aperture angle of the lenses providing spherical wavefronts with a large solid angle. Our study thus underscores once more that the basic tenet of 4Pi microscopy, i.e. the enlargement of the aperture angle by the double-lens system- not the mere production of a two-beam interference pattern- is the actual physical element for improving the axial resolution with opposing lenses.

Another consequence of the more pronounced ‘sphericity’ of the converging illumination wavefronts is the fact that the axial lobe height and the modulation depth of the signal along the optic axis now strongly depends on the lateral extent of the object to be axially separated. This dependence is vividly demonstrated in Fig. 2. Point-like objects can be better separated than stacked planes. Therefore, careful observance of the optical conditions, such as selecting the required cover glass thickness and using the proper embedding medium are more important for imaging the latter. We have shown that these issues can be handled so that even dual-color 3D-images of axially stacked cellular organelles, such as the Golgi apparatus, can be obtained.

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