4Pi-confocal microscopy of live cells

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Abstract

By coherently adding the spherical wavefronts of two opposing lenses, two-photon excitation 4Pi-confocal fluorescence microscopy has achieved three-dimensional imaging with an axial resolution 3–7 times better than confocal microscopy. So far this improvement was possible only in glycerol-mounted, fixed cells. Here we report 4Pi-confocal microscopy of watery objects and its application to the imaging of live cells. Water immersion of 4Pi-confocal microscopy of membrane stained live Escherichia coli bacteria attains a 4.3-fold better axial resolution as compared to the best water immersion confocal microscope. The resolution enhancement results into a vastly improved three-dimensional representation of the bacteria. The first images of live biological samples with an all-directional resolution in the 190–280nm range are presented here, thus establishing a new resolution benchmark in live-cell microscopy. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

The past decades have witnessed the advent of a number of powerful microscopes for biological imaging. Still, far-field light microscopy remains the only method capable of imaging the interior of living cells at the sub-micron scale. Confocal and multiphoton microscopes stand alone in their ability to provide three-dimensional (3D-) information through optical axial sectioning [1]. Whereas, the best lateral resolution of these microscopes ranges between 180 and 300 nm, depending on the wavelength, their finest axial section is only of the order of 500–1000 nm [1,2]. This is particularly unfortunate as far-field light microscopy is a unique method for visualising the relationship between structure and function in live cells. Hence, improving the 3D-resolution in live cell microscopy is a key step towards gaining further insight into the structure–function relationship.

The limited far-field microscopy resolution, although shown to be defeatable [3,4], is ultimately rooted in the phenomenon of diffraction [5]. The fact that the axial resolution is poorer than its lateral counterpart arises from the diminished coverage of the total aperture along the optic axis when focusing with a single lens. 4Pi-confocal
microscopy improves the axial resolution by utilising two opposing objective lenses whose wavefronts are coherently combined to give a greater total aperture [6, 7]. This arrangement yields a point-spread function (PSF) with a main maximum 3–7 times sharper along the optic axis and two undesired side lobes, whose effect can be removed by a linear filter [8]. When further combined with non-linear image restoration, 4Pi-confocal microscopy achieves a 3D-resolution in the 100 nm range, as demonstrated by imaging microtubules and actin filaments in cells [9, 10]. This has recently been confirmed by a similar method, I^2M, that also coherently uses the wavefronts of two opposing lenses both in illumination and detection [11].

Thus far, 4Pi-confocal microscopy and also I^2M have been confined to the imaging of non-living cells. This stems from the fact that the 4Pi-confocal microscope, and even more so the I^2M, reach their best performance with the highest possible aperture angle \( \alpha \), of course. The highest \( \alpha \) of all immersion lenses is found with oil immersion lenses of numerical aperture NA = \( n \sin \alpha = 1.4 \), \( \alpha = 67.3^\circ \). However, oil immersion (\( n \approx 1.51 \)) requires the sample to feature a refractive index \( n \geq 1.45 \) because the refractive index step induces intolerable spherical aberration [12]. This essentially precludes the use of oil immersion lenses for imaging watery specimens (\( n = 1.33 \)) both in 4Pi, but also in confocal microscopy. For 4Pi-confocal microscopy the specimens had to be mounted in glycerol.

High aperture, NA = 1.2, water immersion lenses avoid these spherical aberrations, but as their aperture angle is lower by 5.3°, their suitability for 4Pi-confocal microscopy is not at all obvious. The reason is that according to diffraction theory [13] the main maximum of the PSF scales with \( \sim 1/(\sin \alpha)^2 \), so that lower aperture angles \( \alpha \) result in an increased number of interference side lobes that can no longer be removed.

By studying the focal diffraction maxima of water immersion lenses with isolated fluorescent point objects we recently obtained encouraging evidence that the undesired interference side lobes may be reduced by implementing two-photon excitation in conjunction with confocal detection [14]. In this paper, we report the first water immersion imaging with fundamentally improved axial resolution. Whereas, in regular live cell confocal or multiphoton [15] 3D-imaging axial sections of only 800–900 nm are obtained, we now demonstrate sections as fine as 190 nm. For the first time, this resolution is achieved in live cells.

2. Water immersion 4Pi-confocal microscope

In this section, we briefly summarise the principles of 4Pi-confocal microscopy. Fig. 1(a) shows the geometry of the 4Pi-confocal microscope of type A used here. The pulse train of a compact, diode-pumped Titanium Sapphire-laser (Tiger, Time Bandwidth Products, Zurich, Switzerland) emitting 100 fs pulses at a repetition rate of 100 MHz, and at a centre wavelength of 825 nm, is split by a beamsplitter (BS) into two beams. After travelling different optical pathways of the same pathlength, the wavefronts meet in the common focus of two opposing lenses both in illumination and detection [11].

Confocal detection is realised through an optical fibre. The back projected Airy disk is of the order of the fibre diameter, which is connected to a counting avalanche photodiode (SPCM-131-FC; EG&G Optoelectronics, Vaudreul, Canada). During image acquisition the sample is scanned by a piezo-electric stage (Melles Griot, Cambridge, England) through the focus. The stage has a precision of 10 nm in all directions. The instrument, as well as the confocal detection, is covered by a box to reduce stray-light and potential phase fluctuations due to air currents in the two optic pathways of the 4Pi-confocal microscope.

The back apertures of the opposing objective lenses are illuminated either at an intensity of \( \sim 3 \) or 1–2 mW for the imaged immunolabelled cells and the live bacteria, respectively. The Leica water
Fig. 1. (a) Photograph of a 4Pi-confocal microscope. The illumination wavefront is split at the BS and meets at the common focus of two opposing water immersion lenses. The fluorescence of the sample is measured by a confocal detector. The adjustment and stability control of the flexible right lens is achieved by partial reflection of the transmitted light by a pellicle. The pre-adjustment of the phase is accomplished by a piezo-electrically driven mirror. The 4Pi-confocal microscope is turned into a confocal microscope by closing the right illumination beam with a shutter. (b) The 3D-rendered response of the 4Pi-confocal mode to a sub-resolution fluorescence point-object shows a main maximum and two side lobes (left) which can be removed by deconvolution with a linear inverse filter (centre). The threshold for surface rendering was set to 16% of the global maximum. The 4Pi-confocal 3 point deconvolved, effective PSF (right) has to be contrasted with the confocal mode (below). In the axial direction the point-deconvolved 4Pi-PSF is by a factor of 4.3 sharper than the confocal PSF and more spherical.
immersion lenses feature a NA = 1.2 (Leica Planapo, 63 × 1.2 W korr, Leica, Wetzlar, Germany). In order to obtain optimal resolution with respect to least aberration, the correction collars of the water immersion lenses are adjusted to match the thickness of the coverslip. We therefore select coverslips of thickness 170 ± 2 μm, as the dynamic range of the correction collar on the objective lens limits coverslips to the thickness between 140 and 180 μm.

In order to control the lateral position of the adjustable right objective lens, a pellicle is introduced into the right arm of the microscope, as depicted in Fig. 1a. The pellicle reflects ~10% of the light coming through the left arm. This light has already passed through both objective lenses and is optically magnified onto a CCD-camera. Since the setup is stable at 22 ± 1°C over several tens of minutes, only slight online readjustments of the objective lens have to be made during image acquisition. Except for monitoring the room temperature no active thermostat is used to keep the temperature constant. For the pre-adjustment of the relative phase between the light coming from the left and the right side, meeting in the focus, a phase mirror is implemented in the right arm. By closing one of the two optical paths in the setup, the 4Pi-microscope turns into a confocal microscope. The phase mirror and shutters are also sketched in Fig. 1a.

The 4Pi confocal microscope of type A uses the coherently added wave fronts for illumination and a single focus confocal detection. In the two-photon excitation fluorescence case [16], the effective 4Pi-confocal PSF and with that of single lens accounting for confocal detection $h_{4Pi(A)}(r,z) = |E_1(r/2, z/2) + E_2(r/2, z/2)|^2$. The division by 2 accounts for the requirement of doubling the excitation wavelength for two-photon excitation. $E$ is the normalised electric field in the focal region of the objective lens, calculated at the centre fluorescence wavelength; $h_{4Pi}(r,z) = |E(r,z)|^2$. The interference side lobes stemming from the coherently superimposed fields $E_1(r/2, z/2) + E_2(r/2, z/2)$ are effectively suppressed by two-photon excitation in combination with confocal detection [16]. In water immersion lenses we obtained axial side lobes of ~40% of the main maximum. The axial full-width at half-maximum (FWHM) was 190 nm, as opposed to the 820 nm measured with a single lens.

If the side lobes are lower than 50%, they can be unambiguously removed by a linear one-step deconvolution [8]. This is exemplified in Fig. 1b where the 3D-surface rendered, water immersion 4Pi-confocal PSF (left panel) is displayed. It was recorded at the two-photon excitation wavelength of 825 nm and confocal detection with a back-projected Airy disk corresponding to about the diameter of the maximum emission wavelength at around 580 nm. The measurement was performed by scanning a 110 nm fluorescent polystyrene bead (Nile Red, Molecular Probes, Eugene, OR) through the joint focus of the water immersion lenses.

We measured side lobes of a relative height of 36% of the main maximum, which enables efficient point-deconvolution. The centre panel sketches the inverse filter for deconvolution that mainly consists of three points. The inverse filter takes into account the relative position and height of the lobes and renders a narrow main maximum (right panel). Being fast, point deconvolution is carried out along the optic axis, line by line for each XY-coordinate in the plane. By applying the point deconvolution to the complete 3D-data, an image is obtained with a resolution that is determined solely by the sharp main maximum of the 4Pi-confocal PSF, which acts as the effective 4Pi-confocal PSF [8]. For comparison the panel on the right-hand side (bottom) shows the surface-rendered PSF of the confocal water immersion microscope.

To ensure constructive interference in the focus, the relative phase of the two wavefronts is fine-adjusted using a piezo-mirror. So far, the phase and thus the interference mode has been extracted from the images using the response to structures with a small axial extent. These structures were either part of the imaged specimen, such as microtubules, or added on to the specimen by means of small fluorescent beads. Here, we monitored the relative phase during image acquisition.
acquisition, by means of a thin fluorescent film, coated onto the coverslip. The 4Pi-response of the film is used for an online phase adjustment [17]. The low fluorescent emission of the film does not mask the specimen fluorescent emission. In addition, due to its distinct localisation at the surface of the coverslip it is easily distinguishable from the observed specimen. The fluorescent film can be seen on some of the presented images. Though it is not visible in all pictures, it is present in all 3D data stacks.

3. Specimen preparation

*Escherichia coli* K-12 DH5α were grown overnight to steady state in LB-medium (10 g/l tryptone, 5 g/l yeast extract and 10 g/l NaCl) at 37°C. Membrane labelling was accomplished by adding the vital stain FM 1–43 (Molecular Probes, Eugene, OR) to a final concentration of 0.1 mM from a 2.5 mM EtOH stock solution to the bacterial culture. Staining was performed at room temperature for 2–4 h. Bacteria other than *E. coli* grew in non-sterilised LB-medium at room temperature, which, by providing structures that were unresolvable in confocal microscopy, were also used for imaging. The staining procedure for these bacteria was the same as for *E. coli* but with a final FM 1–43 concentration of 5 μM. To mount the live bacteria, glass cover slips were first coated with poly-L-lysine (Sigma, St. Louis, MO). The coverslips were briefly incubated with 0.1 mM FM 1–43 to produce a thin fluorescent film on the glass surface. After a washing step the membrane labelled bacteria were re-suspended in PBS (45 mM NaH2PO4, 130 mM NaCl, pH 7.8) or LB-medium and placed between two coverslips.

Immunofluorescence staining of microtubules of NIH3T3 cells was performed essentially as described [18]. In brief, cells were fixed for 20 min in ice-cold methanol. After several washing steps staining was performed with a monoclonal anti-α-tubulin antibody and a secondary Oregon Green labelled anti-mouse antibody (Molecular Probes, Eugene, OR). Samples were mounted in a watery mounting medium (100 μM Tris-HCl (pH 8.5), 25% Glycerol) containing 9% Mowiol 4–88 (Hoechst, Frankfurt, Germany) as an anti-bleaching agent.

4. Results

4Pi-confocal microscopy with water immersion lenses is first demonstrated by imaging fluorescently labelled microtubules in the fixed NIH3T3 cells. Fig. 2a,b compares 4Pi-confocal XZ-images taken from the labelled tubulin network with their single lens confocal counterpart. The increase in axial resolution is manifested in the vastly improved ability of the 4Pi-confocal microscope to distinguish microtubules or microtubular bundles along the optic axis.

The images shown in Fig. 2a,b are part of 3D-image stacks, each consisting of 30 XZ-images that were 100 nm apart along the Y-direction. The pixelation of the XZ-images is 39 and 54 nm/pixel in the axial and lateral directions, respectively. The pixel dwell time was 1 ms for both the 4Pi and the confocal imaging mode. The total stack size is $7 \times 5 \times 3 \mu m$ in X-, Y-, and Z-direction, respectively. The confocal mode is achieved by closing the optical pathway of the right arm of the 4Pi microscope. The illumination intensity for the confocal image was doubled, in order to obtain a similar signal strength in both cases.

Fig. 2c,d compares the images of the data stacks of microtubular bundles after volume rendering, as obtained with the 4Pi-confocal (Fig. 2c) and the water immersion single lens confocal (Fig. 2d). The imaged microtubules are presumably single fibres the thickness of which is well below the resolution limit of the 4Pi-confocal microscope. Thus, they can be used to determine the resolution of the microscope itself. As indicated by the arrows in the bottom XZ-image of Fig. 2a, we found an axial FWHM of 190 nm. This should be contrasted with the 820 nm found in the corresponding confocal image. The decrease in FWHM discloses a resolution improvement by a factor of 4.3 in the axial direction. While the resolution in the lateral direction remains unchanged at 280 nm, the superior axial resolution of the 4Pi image is clearly manifested in the data. Whereas, the rendered confocal data stack does not allow any separation,
the 4Pi clearly separates several fibres and reveals their entanglement in space.

As a next step we imaged living *Escherichia coli* bacteria whose membranes were labelled with the vital stain FM 1–43. The gram negative bacterium *E. coli* possesses an outer and a cytoplasmic membrane that are only a few nm apart and therefore not distinguishable. In Fig. 3 a single optical XZ-slice, 4.4 × 2.6 μm, of a small *E. coli* is shown, imaged in the 4Pi-confocal (Fig. 3a) and single lens confocal (Fig. 3b) mode. The 3D-data stack has a lateral depth of 3 μm. The comparison reveals the limitation of confocal microscopy. Due to the axially elongated spot the confocal microscope artificially overemphasises those parts of the membrane that are oriented parallel to the optic axis. Moreover, the confocal microscope is not able to recognise the membrane as a distinct feature surrounding the cell. In contrast, the 4Pi-confocal image discloses the outline of the cell.

The fundamentally improved resolution of the 4Pi-confocal microscope is best recognised in the intensity-line profile taken from the centre of the respective images. Whereas, the 4Pi-confocal image (Fig. 3a) resolves a cell diameter of 0.7 μm, the confocal recording (Fig. 3b) is completely unable to resolve the cell membrane. In fact, it is impossible to recognise the bacterium on the basis of the confocal data.

Fig. 4 shows 4Pi- and single lens confocal XZ-sections through an *E. coli* which has almost finished its dividing process. Similar to Fig. 3 the more than 4-fold improved spatial resolution along the Z-axis results in a much better representation of the bacterium. On the right-hand side of the 4Pi-confocal picture a fine vertical shadow can be recognised, as a trace of the thin film on the cover slip with which we adjusted the initial relative phase of the two counter-propagating focused wavefronts.

Next, we imaged agglomerated fluorescently labelled *E. coli* distributed over 8 μm in the Z-direction. This is a comparatively large distance along the optic axis. Fig. 5 shows a portion of a
single XZ-slice, 2.0 × 8.0 μm, taken with the 4Pi-confocal (Fig. 5c) and the single lens confocal (Fig. 5d) microscope, respectively. In the confocal picture (Fig. 5d) the fluorescence signal is smeared out over the agglomerated bacteria so that hardly any bacterium can be recognised. The corresponding axial intensity plot in (Fig. 5b) does not aid in the recognition of single cells either. In contrast, the 4Pi-confocal image (Fig. 5c) permits the distribution of the cells to be determined. Fig. 5e is a sketch of the membranes identified by the 4Pi-confocal microscope as a guide to the eye. As illustrated in the axial intensity plot in (Fig. 5a), in the 4Pi-image each membrane contributes to a distinct fluorescence peak.

An important result is that for the 8 μm axial scan, the relative phase of the PSF remains unchanged. This is not so when imaging glycerol mounted, fixed cells with oil immersion lenses. The reason is that the refractive index step between the cover glass and the glycerol induces a linear phase change with increasing penetration depth [19]. To ensure the same relative phase of the counter-propagating wavefronts this phase change has to be counteracted by an external active phase compensation [9]. Fig. 5c is important since it evidences that once installed, 4Pi-confocal imaging of clear watery specimens is possible without further active phase change. In that sense, water immersion 4Pi-confocal microscopy actually imposes fewer precautions than its oil immersion counterpart.

In addition, in oil immersion 4Pi-confocal and I5M microscopy, spherical aberration limits the total thickness of glycerol-mounted specimens to a maximum of 20–30 μm [19]. Featuring virtually the

Fig. 3. Live Escherichia coli cell imaged with the 4Pi-confocal (a) and the confocal modes (b). Note that for the 4Pi-confocal image, the fluorescently labelled membranes can be distinguished in the axial direction, which is not the case in the confocal image. The axial membrane distance is 0.74 μm. The plotted XZ-images have a size of 4.4 × 2.6 μm. The intensity line profiles, taken from the centre of the respective cells show quantitatively the superior resolution of the 4Pi-confocal mode.

Fig. 4. Dividing Escherichia coli; shown are single optical XZ-slices of the live cell recorded by the water-immersion 4Pi-confocal microscope (a) and its confocal counterpart (b). The images were extracted out of 3D-image-stacks of 3.1 × 5.7 × 2.0 μm in axial (Z) and X-, Y-direction, respectively. The fine line-shaped vertical shadow in the 4Pi-image (arrow) is due to the thin fluorescent film on the cover slip, with which the relative phase of the interfering wavefronts was initially adjusted.
same water-like refractive index of $n \approx 1.33$ on both sides of the cover slip, water immersion 4Pi-confocal imaging neither requires phase compensation, nor confines the maximum thickness of the specimen. For predominantly clear specimens, such as *E. coli*, the maximum allowable specimen thickness will probably be given by the free-working distance of the NA = 1.2 water immersion lenses, which is 200 µm. Again, this is larger by a factor of 3 than that of 1.4 oil immersion lenses.

To further demonstrate the capability of the 4Pi-confocal microscope to image living cells with superior 3D-resolution we labelled the membrane of bacteria growing in the non-sterilised growth medium. A 4Pi-confocal (Fig. 6a) and a confocal XZ-image (Fig. 6b) of an agglomeration of these bacteria are shown. Again, the 4Pi-confocal microscope sharply resolves the bacterial membranes. In contrast, in the confocal image the shape and membrane of the bacteria are smeared out and unrecognisable. Contrary to the confocal microscope, a gap in between the bacteria is discerned in the 4Pi-confocal image (see arrow in Fig. 6).

Fig. 7a shows an XZ-image of a 1.7 µm thin sample. The distance between the cover slips was determined from the distance between the signal from the fluorescent film on the cover slips. Its intensity is minimal compared to the high signal of the labelled bacterial membrane. Due to the large and axially elongated PSF, in the confocal microscope, but not in the 4Pi, the signal from the bacterial membranes overwhelms that from the film (Fig. 7b). To give a 3D impression of the labelled cells, the 3D data-stack of the 4Pi-confocal image is rendered in Fig. 7c. Its size is $3.9 \times 3.9 \times 3$ µm in $XZY$-directions, respectively. The surface-rendered picture reveals the 3D-shape of the fluorescently labelled membrane along with the thin fluorescent film on the cover slips.

Fig. 5. Conglomeration of live *E. coli* stained with FM 1–43. The diagram indicates the position and size of the imaged cells. The diagram has been deduced from the 4Pi image. The cells have been imaged in both, the 4Pi-confocal and the confocal modes. The axial intensity plots are taken at the indicated positions. The $XZ$-images have an extent of $2.0 \times 8.0$ µm. Note that for the 8 µm axial scan no phase compensation was needed. The corresponding 3D-stack has a lateral depth of 3.0 µm.
5. Discussion and conclusion

The attained improvement of axial resolution by a factor of 4.3 constitutes a new resolution benchmark in the imaging of live specimens. Our study reveals an axial resolution of 190nm (FWHM), achieved with a two-photon excitation wavelength of 825 nm. This value should be contrasted with the 820 nm (FWHM) axial resolution obtained with single lens two-photon confocal microscopy, which is a high-end imaging mode in terms of resolution in watery specimens.

The 4Pi-confocal microscope leaves the 280 nm lateral FWHM of the effective PSF unchanged. However, as the axial FWHM of 190 nm is now closer to its lateral counterpart the 4Pi-confocal PSF renders more faithful 3D-images. In contrast, the elongated PSF of the confocal microscope leads to characteristic artefacts in the axial section. The confocal data overemphasise the brightness of those parts of the sample that are oriented along the optic axis. More importantly, axial features in the sub-micron range are not resolved. Systematic comparison of the 4Pi-confocal and single lens confocal images in Figs. 3–7 proves that the 4Pi-
confocal microscope leads to fundamentally better images of the live cells, throughout. The superiority of the axial over the lateral resolution does not violate Abbe's diffraction limit [5] but stems from the fact that with two opposing lenses the total 3D-aperture is now better covered along the optic axis than in the lateral direction. The linear filter used for removing the axial lobes is fast and performed during data acquisition. It should not be confused with the commonly applied, powerful, and computationally extensive image restoration procedures [20–22]. However, if applied, offline non-linear image processing can further improve the spatial resolution by about 50% in all directions [9,23].

While the results reported herein represent a substantial step in the development of far-field microscopes, some technical aspects remain to be improved in the future. Whereas, the application of a thin fluorescent film on the cover slips turns out to be a good solution, it would be desirable to extract the phase from random object data, which is conceivable. A further significant improvement of the technique in terms of image acquisition speed is anticipated from a beam-scanning version or a multi-focal version of 4Pi-confocal microscopy. With such systems the current imaging speed of typically 30–60 s per AXZ-slice will be reduced at least by an order of magnitude. The higher imaging speed will also allow us to extend 4Pi-confocal microscopy of live cells to the imaging of more complex cells and perhaps tissue sections. Developments of this kind are underway in our laboratory.

In conclusion, we have reported the realisation of water immersion 4Pi-confocal microscopy and provided the first 4Pi-confocal images of cells, here bacteria, in their native watery environment. The obtained 3D-resolution of 280 and 190 nm in the lateral and axial directions, respectively, sets a new lower benchmark in the 3D-imaging of live cells. In fact, the imaging data presented herein constitute, to our knowledge, the best-resolved three-dimensional far-field images in live biological microscopy.

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