

4Pi Microscopy

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INTRODUCTION

The axial (z -) resolution of any fluorescence microscope using a single lens is limited by diffraction to $>500\text{ nm}$. While a modest improvement by up to a factor of 2 may be achieved by mathematical deconvolution, a substantial improvement of the axial resolution requires a radical change of the physics of imaging. Since the 1990s, two families of methods have evolved that accomplished substantially improved axial resolution in three-dimensional (3D) imaging. The first family, comprising 4Pi microscopy and I²M, coherently combines the aperture of two opposing lenses (Hell and Stelzer, 1992a, 1992b; Gustafsson *et al.*, 1995, 1999; Egner and Hell, 2005). The second family, of which stimulated emission depletion (STED) microscopy (Hell and Wichmann, 1994; see also Chapter 31, *this volume*) is the most established member, exploits photophysical or photochemical properties of the dye to break the diffraction barrier. Using two lenses for STED, the axial resolution was improved down to 30 to 50 nm (Dyba and Hell, 2002; Dyba *et al.*, 2003). However, as STED is still in its infancy and, unlike I²M and 4Pi microscopy, relies on the specific properties of the dye, it is not surprising that the majority of recent 3D-imaging applications showing axial super-resolution in (live) cells have been carried out with the 4Pi microscope (Egner *et al.*, 2002b, 2004; Gugel *et al.*, 2004).

4Pi microscopy coherently illuminates the sample through two opposing lenses. Constructive interference of the counterpropagating spherical wavefronts narrows the main focal maximum of the excitation light in the z -direction, and this forms the basis for a 3- to 7-fold improved axial resolution. Unfortunately, the interference of spherical wavefronts also produces so-called sidelobes¹ that are located at about half a wavelength above and below the focal plane. Moreover, these primary lobes are accompanied by weaker periodic lobes that increase in height with decreasing semi-aperture angle α . The lobes produce ghost images that have to be removed by mathematically postprocessing the 3D data. The accuracy with which these ghost images can be removed depends on the relative height of the sidelobes compared to the main peak. As a rule of thumb, the primary lobe height should never exceed $\sim 50\%$ of the main focal spot. Unfortunately, available immersion lenses all feature $\alpha < 68^\circ$ so that the primary lobe height ranges from 60% to 70% of the main peak. Additional optical techniques for lobe suppression thus have to be implemented, of which the most effective are:

- **Confocal detection:** Fluorescence emitted from out-of-focus planes is detected less efficiently, thus suppressing fluorescence detection from the sidelobes.
- **Two-photon excitation (2PE):** The quadratic dependence of the excitation efficiency on the illumination intensity lowers the excitation sidelobe height relative to the central peak. Additionally, due to the longer wavelength of the near-infrared light used (Denk *et al.*, 1990), the sidelobes are shifted away from the focal plane and are better suppressed by the pinhole.²
- **Combined interference of the excitation and the detection wavefront pairs:** The wavelength disparity leads to different axial positions of the excitation and the detection sidelobes. As a result, fluorescence originating from the excitation sidelobes is weakened by incomplete detection. 2PE entails a pronounced and very useful wavelength disparity (Hell and Stelzer, 1992a, 1992b; Gugel *et al.*, 2004).

The potential of these three lobe-suppression techniques was already recognized in the early stages of 4Pi microscopy (Hell and Stelzer, 1992a, 1992b). In fact, the use of 2PE together with confocal detection has been the method of choice ever since. The third technique is more difficult to realize. The optical setup must be designed such that not only the excitation light interferes at the sample (Type A 4Pi), but also the detection light interferes at the point of detection (Type C 4Pi). Initially, Type C 4Pi-microscopy could be demonstrated only for scattered light, where excitation and detection wavelengths are the same and the implementation is much simpler (Hell *et al.*, 1994). A feasible realization for fluorescence that exploits the suppression potential of this technique is shown in a recent publication (Gugel *et al.*, 2004). The 2PE Type C mode combines all three mechanisms and thus features the lowest achievable sidelobes. This attribute greatly simplifies the image restoration process. Additionally, Type C 4Pi microscopy obtains a $\sim 30\%$ better resolution than its Type A counterpart.

In contrast to 4Pi microscopy, I²M uses planar standing waves for fluorescence excitation; in fluorescence detection, the coherent addition of spherical waves is performed just as in a 4Pi Type C microscope. In I²M, the joint action of illumination and detection interference is mandatory because other mechanisms of lobe suppression are absent here. This results in relatively high sidelobes making image restoration more difficult (Bewersdorf *et al.*, 2005; see Appendix, *this chapter*).

¹ Actually they are located above and below, rather than to either side.

² On the other hand, 2-photon 4Pi can only be implemented optimally if the total optical lengths of each side of the interferometer are equal, $\pm 10\mu\text{m}$.

Apart from the sidelobe reduction, 4Pi microscopy has also undergone significant progress in terms of increasing the recording speed. Due to the smaller focal volume of excitation, less total fluorescence is excited at a given time point. Additionally, the axially narrower focal spot requires finer sampling. The deconvolution required for the lobe removal also sets a limit to the acceptable minimum signal-to-noise ratio (S/N). Thus, to achieve an adequate S/N, the recording time may easily take longer by the factor by which the volume resolution is reduced typically by a factor of two. Lengthened recording times may be counteracted by investigating smaller regions. Another powerful approach is to parallelize the recording process by using many 4Pi foci in parallel, as in a multifocal multi-photon microscope (MMM, see Chapter 29, *this volume*) (Bewersdorf *et al.*, 1998). In fact, the combination of MMM with 4Pi microscopy has resulted in a fast axially super-resolving microscope (Egner *et al.*, 2002b, 2004). In this chapter we give an overview of modern beam-scanning 4Pi microscopy based on the two most recent realizations: the MMM-4Pi as described by Egner and colleagues and the Leica TCS 4Pi, which is a compact Type C 4Pi microscope that has recently become commercially available.

THEORETICAL BACKGROUND

The Point Spread Function

The imaging capabilities of a microscope, such as the resolution, are quantified by the effective PSF $h(\vec{x})$, that is, the response of the microscope to a fluorescent point. The effective PSF can be calculated as the product of the excitation PSF and the detection

PSF, $h_{exc}(\vec{x}) h_{det}(\vec{x})$. The excitation PSF $h_{exc}(\vec{x})$ is proportional to the illumination intensity squared in the focal region and, apart from a constant factor, can be written as

$$h_{exc}(\vec{x}) = |\vec{E}_{ill}(\vec{x}) + \hat{M} \cdot \vec{E}_{ill}(\hat{M} \cdot \vec{x})|^4$$

with

$$\hat{M} := \begin{pmatrix} 1 & 0 & 0 \\ 0 & 1 & 0 \\ 0 & 0 & -1 \end{pmatrix}$$

where $\vec{E}_{ill}(\vec{x})$ denotes the focal light field of the illumination by a single lens and $\hat{M}$ is a transformation matrix accounting for the counterpropagation of the fields in the 4Pi microscope. 2PE is incorporated by the forth power of the illumination light field (instead of the quadratic power for single-photon excitation).

For the detection PSF $h_{det}(\vec{x})$ that expresses the spatial distribution of the detection efficiency, the finite pinhole size has to be taken into account. $h_{det}(\vec{x})$ also depends on the 4Pi mode:

$$h_{det,4Pi-A}(\vec{x}) = |\vec{E}_{det}(\vec{x})|^2 \otimes p(\vec{x}) \quad \text{for Type A}$$

$$h_{det,4Pi-C}(\vec{x}) = |\vec{E}_{det}(\vec{x}) + \hat{M} \cdot \vec{E}_{det}(\hat{M} \cdot \vec{x})|^2 \otimes p(\vec{x}) \quad \text{for Type C}$$

In analogy to the illumination light field and due to reasons of symmetry, $\vec{E}_{det}(\vec{x})$ represents the normalized amplitude of the detection of the fluorescence light field emanating from the focal region. To consider the role of the pinhole, $\vec{h}_{det}(\vec{x})$ is convolved with $p(\vec{x})$, denoting the pinhole function is effective in the focal region. Using a vectorial focusing theory (Richards and Wolf, 1959) $\vec{E}_{ill}(\vec{x})$ and $\vec{E}_{det}(\vec{x})$ can be calculated thoroughly. Theoretical PSFs for Type A and C 4Pi microscopy are displayed in Figure 30.1. Comparing the extent of the central maxima of the 4Pi PSFs with that of the

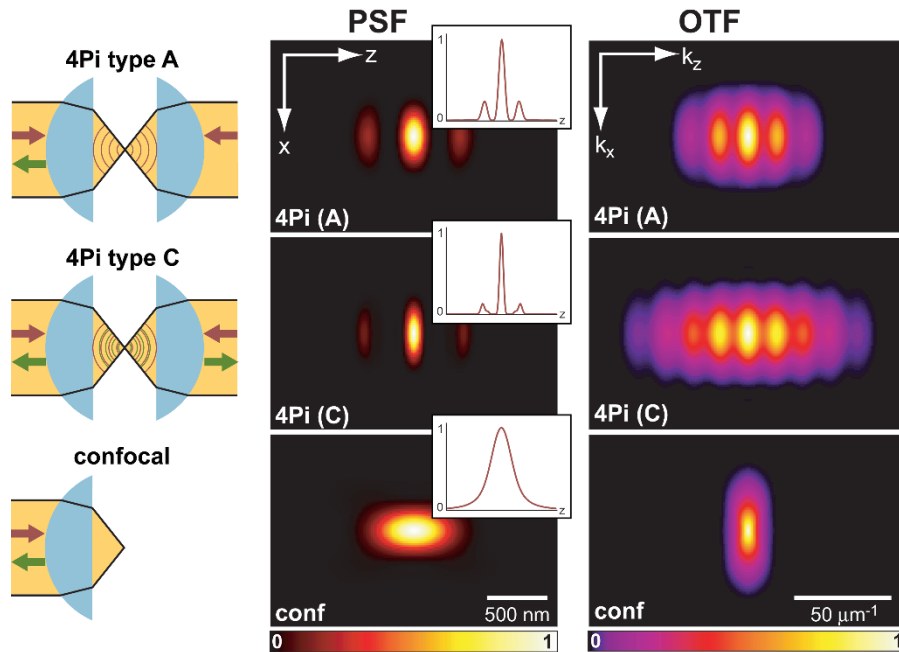


FIGURE 30.1. The PSFs and OTFs of two-photon excitation 4Pi microscopy. In Type A 4Pi microscopy, the sample placed between two coverslips is coherently illuminated by two opposing objective lenses focusing a pulsed infrared laser beam into the same spot. The 2PE fluorescence is collected from a single side and focused onto a (variable) confocal pinhole. While the excitation mode is the same, in Type C 4Pi microscopy, the fluorescence is collected by both lenses and coherently combined at the detector. As can be seen from the calculated PSFs and the z -responses shown as insets, the 4Pi microscope (water-immersion lens 1.20 NA, $\lambda_{0,exc} = 870$ nm, $\lambda_{0,det} = 510$ nm, pinhole diameter of 1 Airy disk) features a four- to sevenfold enhanced axial FWHM compared to confocal microscopy ($\lambda_{0,exc} = 488$ nm, $\lambda_{0,det} = 510$ nm, pinhole diameter of 1 Airy disk). Due to the combination of the interference in excitation and detection, the Type C mode exhibits lower sidelobes and by a ~30% increased axial resolution compared to Type A. The superior resolution of 4Pi microscopy also becomes evident in the much larger support of the 4Pi-OTFs as compared to the confocal OTF. Due to the low sidelobes in the PSF, the 4Pi-OTF is not only free of missing frequencies but also of weak amplitudes within the region of support.

standard confocal PSF makes the resolution increase evident. The sidelobes located $\sim \lambda_{0,exc}/2$ away are also visible.

In the case of the MMM-4Pi, the PSF has to be modified in order to take into account the multiple foci and the charge-coupled device (CCD) camera detection. However, the differences between the PSFs of a single focus Type A 4Pi microscope and the actual MMM-4Pi instrument are almost negligible (Egner *et al.*, 2002b).

The z-Response and the Axial Resolution

4Pi microscopy improves the resolution only along the optic axis. The lateral resolution remains, strictly speaking, the same. However, one has to realize that the fundamental reduction in the thickness of the optical sections also yields a significantly improved perception of details in the focal plane, almost as a beneficial side effect. This is because details that are only slightly (35–75 nm) away from the focal plane are suppressed significantly better than in a confocal microscope, in which a similar reduction requires a displacement of >250 nm.

The z -response of an infinitely thin fluorescent layer, $I_z(z) = \iint h(\vec{x}) dx dy$, is a good benchmark for the axial resolution. The insets in Figure 30.1 show the z -responses for Type A and Type C 4Pi microscopes compared with that of the standard confocal system. For typical imaging conditions, the height of the sidelobes in 2PE Type A 4Pi microscopy remains well below 50%; for the Type C it is lowest. Importantly, the sidelobes increase dramatically in height for decreasing aperture angles. Typically, one uses immersion lenses of a numerical aperture (NA) of 1.4 (oil), 1.35 (glycerol), and 1.2 (water), featuring semi-aperture angles of $\alpha = 67^\circ$, 68° and 64° , respectively. In 4Pi microscopy, the aperture angle has only a marginal impact on the axial width of the central spot. Because it is an interference phenomenon of the two counterpropagating waves, the z -width of the central spot scales predominantly with the wavelength in the embedding medium. For $\lambda_{0,exc} = 870$ nm, $\lambda_{0,det} = 510$ nm, $n = 1.334$ (water immersion) and an angle α of $\sim 65^\circ$, axial resolutions of ~ 150 nm (Type A 4Pi) and ~ 105 nm (Type C 4Pi) are reliably obtained.

The Optical Transfer Function

Representing the Fourier transform of the PSF, the OTF indicates which spatial frequencies of the sample are transmitted by the microscope to form the image. A poorly transmitted spatial frequency means that two objects separated by the corresponding distance (the reciprocal value of the frequency) are blurred into each other in the image. The periodic pattern of the sidelobes of the 4Pi microscope represents such a correlation in space. The more pronounced the sidelobes in the PSF are, the lower the OTF amplitude at the matching spatial frequency is. The removal of the ghost images in the image data by deconvolution methods is in fact an amplification of the weakly transmitted frequencies so that the final OTF becomes smooth and contiguous. The more these amplitudes are masked by noise the more difficult is their reconstruction. Therefore, by analyzing the frequency content of the OTF and the concomitant noise level presented by an object, one learns whether a microscope and the imaging of a particular object are vulnerable to artifacts. The theoretical OTFs for Type A and Type C 4Pi together with their confocal counterpart are shown in Figure 30.1. The 4Pi-OTFs are contiguous; there are no missing gaps or strongly suppressed regions. Compared to the standard confocal

OTF, the 4Pi-OTF features a 4- to 7-fold expanded region of support.

MULTI-FOCAL MULTI-PHOTON MICROSCOPY-4Pi MICROSCOPY

The MMM-4Pi microscope (Egner *et al.*, 2002a) has been designed to increase the recording speed compared to a single-beam 4Pi microscope. It consists of the two subunits sketched in Figure 30.2. The head of the MMM-4Pi includes the beam-splitter and the objective lenses that are illuminated through balanced interferometric arms. Axial scanning is performed by moving the sample along the optic axis (z) as in standard confocal microscopes. The multi-focal beam scanning arrangement is shown in the lower part of Figure 30.2. The beam of a mode-locked titanium:sapphire (Ti:Sa) laser is split by an array of microlenses, producing an $n \times m$ array of beamlets that, after passing through a dichroic mirror, are focused onto an array of pinholes. The pinholes act as point sources for creating the 4Pi illumination spots in the sample. After passing the pinholes, each beamlet is collimated by an achromatic lens and directed towards a rapidly oscillating galvanometric mirror. The following lenses ensure that the galvanometric mirror is imaged into the entrance pupils of the objective lenses. The rapid tilt of the mirror results in lateral scanning of the illumination 4Pi foci in the x -direction; this is the standard scanning procedure found in state-of-the-art confocal beam scanners. Scanning of the beamlets in the y -direction is accomplished by translating the combined

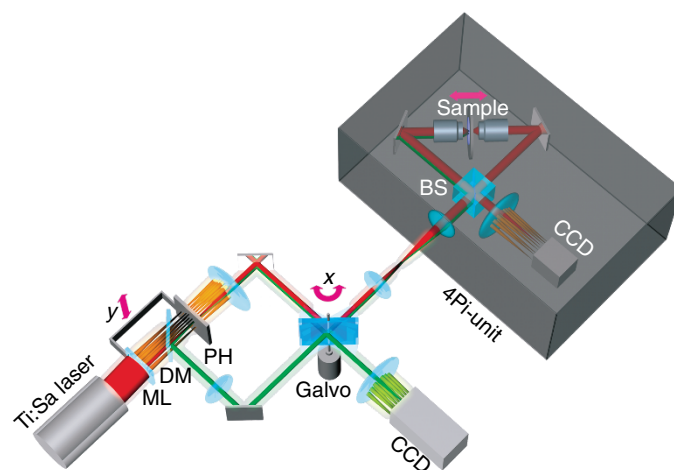


FIGURE 30.2. Scheme of the MMM-4Pi. An array of microlenses (ML) divides the laser beam of a Ti:Sa laser into an array of beamlets. Focused onto a pinhole array (PH), the emerging cleaned beamlets act as point sources for the 4Pi illumination. The beamlets are deflected by the scan mirror (Galvo) and directed towards the 4Pi unit. The beam-splitter (BS) splits up the beams and the beamlets are focused into the sample by both objective lenses. The small CCD detector in the 4Pi unit that detects laser light transmitted through both objective lenses serves as an alignment tool. The fluorescence light is collected by the left objective lens and imaged back onto the pinholes. A dichroic mirror (DM) separates the fluorescence light from the laser light and directs the fluorescence towards the CCD camera. Axial scanning is performed by moving the sample stage along the optic axis. The lateral scan is performed by a rapid tilt of the galvanometric mirror in conjunction with a slower, coordinated movement of the microlens–pinhole array unit. By reflecting the detection light at the backside of the galvanometric mirror, the fluorescence foci are scanned across the CCD camera.

microlens and pinhole array. The fluorescence is collected by the objective lens on the left-hand side and deflected by the galvanometric mirror in order to be back-imaged onto the pinhole array. The diameter of the pinholes corresponds to 1.25 times the diameter of the back-projected Airy disk (i.e., the diameter of the first dark ring). This slight confocalization allows the suppression of the axial sidelobes and also reduces the residual aberrations stemming from the microlenses. After the filtering by the pinholes, the fluorescence light is reflected by the long-pass dichroic mirror towards the reflective backside of the galvanometric mirror. The tilt movement of the latter scans the array of fluorescence beamlets across a CCD camera. Scanning the fluorescence beamlets with the backside of the galvanometric mirrors locks the scan of the excitation foci with that of fluorescence (Brakenhoff and Visscher, 1992). n and m range between 4 and 8, so that a total of $N = n \times m = 16$ to 64 parallel 4Pi foci is typical. Each of them usually performs $\sim 4 \times 4 \mu\text{m}^2$ scans resulting in a field of view of up to $\sim 32 \times 32 \mu\text{m}^2$.

The speed of the MMM-4Pi depends on the degree of parallelization N . The parallelization in turn depends on the available laser power. The average power of 1 to 2 mW in each 4Pi spot leads to a focal peak intensity of $\sim 80 \text{ GW/cm}^2$, which is lower than the $\sim 200 \text{ GW/cm}^2$ considered as an upper limit with $\sim 200 \text{ fs}$ pulses (König et al., 1999). As a mode-locked Ti:Sa laser emits 1 to 2 W of average power, a parallelization by 1000 foci rather than by $N = 16$ to 64 appears to be possible. At present, such a degree of parallelization is precluded by the losses connected with the “cleaning” of the laser output and clipping of the beamlet profile by the pinhole array.

A fundamental problem of any parallelized 3D microscope is the potential crosstalk between neighboring illumination and detection foci. If present, crosstalk deteriorates the axial resolution of the system by re-enforcing specific planes above and beneath the focal plane (Egner and Hell, 2000; Egner et al., 2002a). It was found that in an MMM-4Pi featuring a quadratic grid arrangement, the 4Pi-foci can be brought as close as $3.5 \lambda_{\text{exc}} \approx 2.8 \mu\text{m}$ without inducing significant crosstalk. Therefore, parallelization does not compromise the resolution of a properly designed MMM-4Pi setup (see also Chapter 29, *this volume*).

Space Invariance of the Point Spread Function

To assure accurate image deconvolution, the PSF must be the same throughout the recorded volume. In 4Pi microscopy, this issue is particularly relevant because the phase difference between the two interfering wavefronts has to be kept constant. A (random) change in phase would mean that the PSF varies greatly in the sample. While the envelope of the 4Pi-PSF usually does not significantly change for thin cell layers, a change in phase difference radically alters the interference of the two beams and hence the structure of the 4Pi spot. The following issues are relevant:

- The MMM-4Pi and the Leica TCS 4Pi are beam-scanning systems. To ensure the invariance of the PSF over the whole field of view, the two interferometric arms of the 4Pi microscope are constructed symmetrically. Proper adjustment ensures that the phase is constant over a field $> 30 \mu\text{m}$, as can be seen in Figure 30.3(A–C) (Egner et al., 2002a).
- Slight differences in the refractive index of immersion and mounting medium lead to a z -dependent phase. For the refractive index mismatches typically encountered in the sample, it has been shown that this relationship is linear over a large distance (Egner et al., 1998). If the magnitude of this distortion

is known, a linear change in the length of one of the 4Pi arms with changing z -position of the sample can correct for this phase shift during scanning.

- Irregular changes of the refractive index within the specimen also influence the phase difference between the interfering wavefronts.

To explore whether these conditions can be fulfilled, 3D stacks of (mammalian) *Vero* cells were recorded with water immersion lenses (Egner et al., 2004). The cells were grown on a coverslip coated with Oregon Green and then covered with a similar coverslip so that they were essentially sandwiched between two ultrathin fluorescent layers. To correct for the difference in the refractive indices of the aqueous mounting medium (1.34) and water (1.33) used as immersion medium, the phase had to be adjusted linearly with the z -position. Figure 30.3(D) shows an xz -section of a typical 3D data stack containing a part of the nucleus. The xz -section through the layers on the coverslips and the resulting z -profile disclose the quality of the interference between the two wavefronts. Profiles are extracted from three different sites in the nuclear region, in which the local variations in refractive index are strongly pronounced: completely outside the nucleus, at the nuclear periphery, and straight through the nucleus [Fig. 30.3(E)]. Although slight changes become apparent, the phase does not notably change with the axial translation. This can be inferred from the comparison between the profiles in the same line, pertinent to the different coverslips. Comparison of the profiles from the same coverslip reveals that the presence of the nucleus introduces aberrations leading to elevated sidelobes, but again the changes in refractive index are not pronounced enough to distort the PSF of the 4Pi microscope. In the perinuclear region, the sidelobe height is lower than the critical value of 50% of the central peak, but if the nucleus is directly involved, the lobes are slightly elevated. The upper profile of Figure 30.3(E) also shows that the refractive index challenges are relaxed when largely omitting the nucleus–cytosol interface.

Live Mammalian Cell 4Pi Imaging

In order to further substantiate the applicability of 4Pi microscopy to living mammalian cells, the distribution of two Golgi marker enzymes labeled by EGFP inside living *Vero* cells have been imaged at 32°C with the MMM-4Pi microscope. The marker enzymes were UDP-galactosyl:glycoprotein,1,4- β -galactosyl transferase (GalT), which is highly enriched in the middle and trans Golgi membranes and 3'-phosphoadenylyl-sulfate:uronyl-2-O-sulfotransferase (2-OST) which is a *cis* Golgi marker.

The analysis of series of subsequent 3D recordings of the GalT-EGFP distribution in a live *Vero* cell at 32°C with the MMM-4Pi showed that the movement of the living Golgi apparatus does not pose particular challenges. The slight morphological changes observed between two subsequent recordings occur on a much larger time scale than the scanning of the 4Pi-PSF across each position in the cell. Thus, they did not compromise the space invariance of the PSF and the slight changes did not preclude the proper deconvolution of the data to achieve a 3D resolution of $\sim 100 \text{ nm}$. In addition, the repeated recording of the Golgi apparatus did not show a significant reduction in image brightness. Therefore, photobleaching of EGFP was not an issue in these samples. The cells remained viable even after prolonged mounting in the custom-built chamber for several days. The comparatively large space of $175 \mu\text{m}$ between the two coverslips and the addition of a suitable air/ CO_2 mix is adequate to maintain division and propagation without apparent degradation.

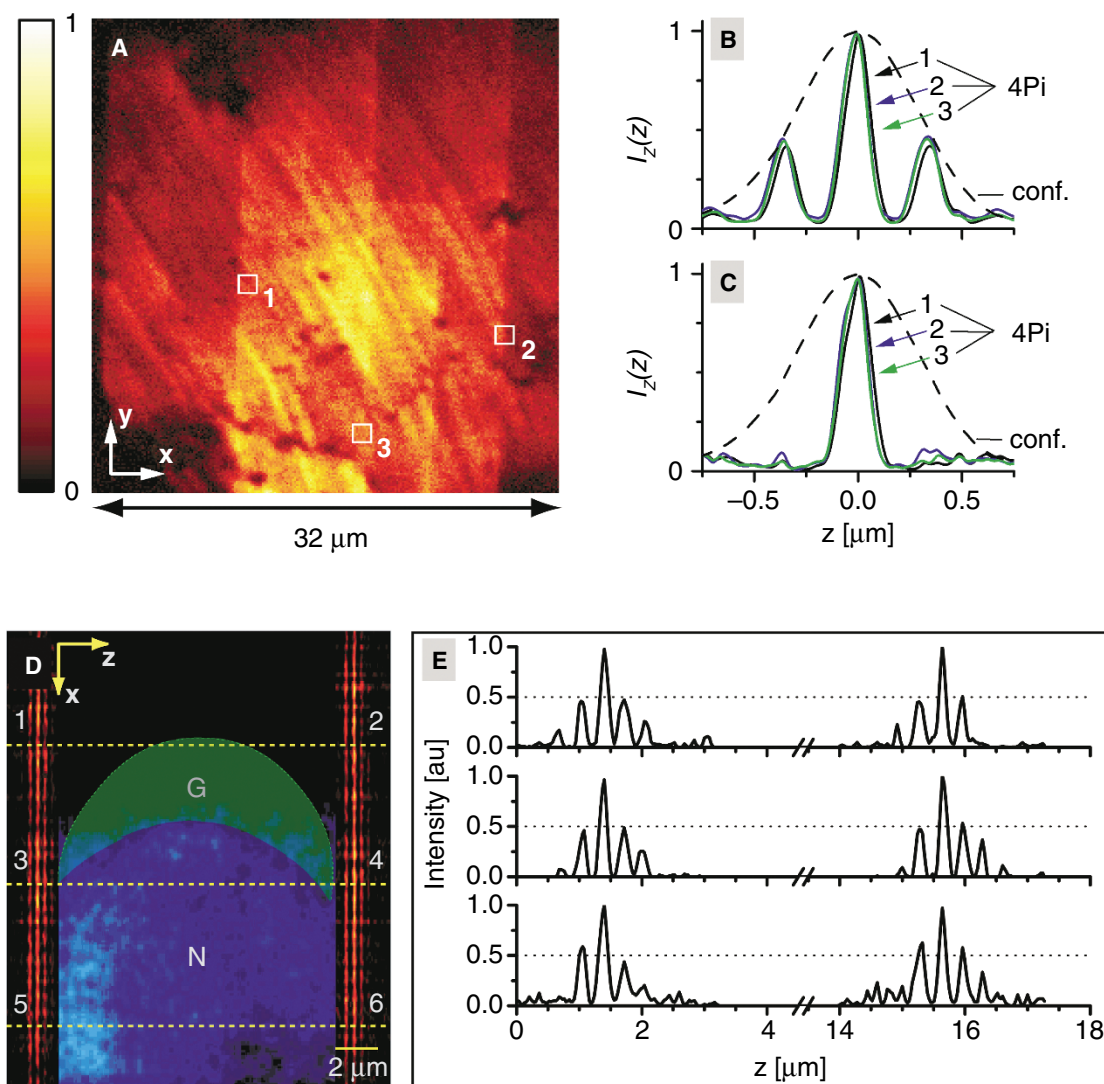


FIGURE 30.3. Basics of MMM-4Pi imaging. (A) xy image of a fluorescent polydiacetylene monolayer taken from a 3D stack. The chessboard appearance is due to the different intensities in the neighboring 4Pi foci; the tilted stripes are inherent to the polydiacetylene monolayer. (B) Axial intensity profiles, $I_z(z)$, through the 3D stack exhibiting a sharp maximum and two lobes, recorded at three different coordinates (x,y) . The spatial invariance of the three profiles indicates that the 4Pi-PSF is constant over the whole field of view, irrespective of the microlens used. Fast linear one-step deconvolution can be applied throughout the field of view to remove the sidelobes (C). (D) xz -section recorded at the periphery of the nucleus of living *Vero* cell sandwiched between two coverslips each thinly coated with a fluorescence layer. The nucleus (N) is highlighted by Hoechst counterstaining and outlined; the region where the Golgi resides (G) is briefly sketched. The cell is significantly larger than the whole xz -section. The three vertical lines on either side (orange) are due to the interference between the counterpropagating focused fields, representing the main maximum of the 4Pi-PSF and the two axially offset sidelobes. (E) Enlarged intensity profiles of the axial responses of the microscope to the thin fluorescent layers, revealing the height of the lobes and the relative phase in greater detail. Outside and at the edge of the nucleus, the primary lobes are <50%. Focusing through the nucleus slightly increases the lobes and induces a noticeable but comparatively small phase shift. The parallelism of the orange lines in the displayed xz -section indicates that the cell does not gravely distort the interference between the wavefronts.

The inset in the upper left-hand corner of Figure 30.4(A) displays a conventional overview xy -image of the GalT-EGFP-labeled Golgi apparatus located close to the nucleus counterstained with the fluorophore Hoechst. The central panel of Figure 30.4(A) displays the corresponding 3D surface-rendered image at 100 nm resolution. The 3D image clearly reveals ribbons, fractioned stacks, as well as smaller tubular and vesicular subcompartments. Furthermore, protrusions and invaginations in the 200 nm range can be discerned. Figure 30.4(B) shows a typical distribution of 2-OST-EGFP in interphase cells. This image also displays a convoluted structure of the Golgi apparatus, similar to that observed for GalT. We think that in the near future, the discrimi-

nation of intra-Golgi localization of 2-OST and GalT will be possible by applying two-color 4Pi microscopy.

TYPE C 4Pi MICROSCOPY WITH THE LEICA TCS 4Pi

The Leica TCS 4Pi (Gugel *et al.*, 2004) consists of a commercially available confocal microscope (Leica Microsystems Heidelberg, Mannheim, Germany) combined with a 4Pi unit. The 4Pi unit is tightly mounted to the microscope turret to maintain all the capabilities of the scanning confocal unit in the microscope body (Fig.

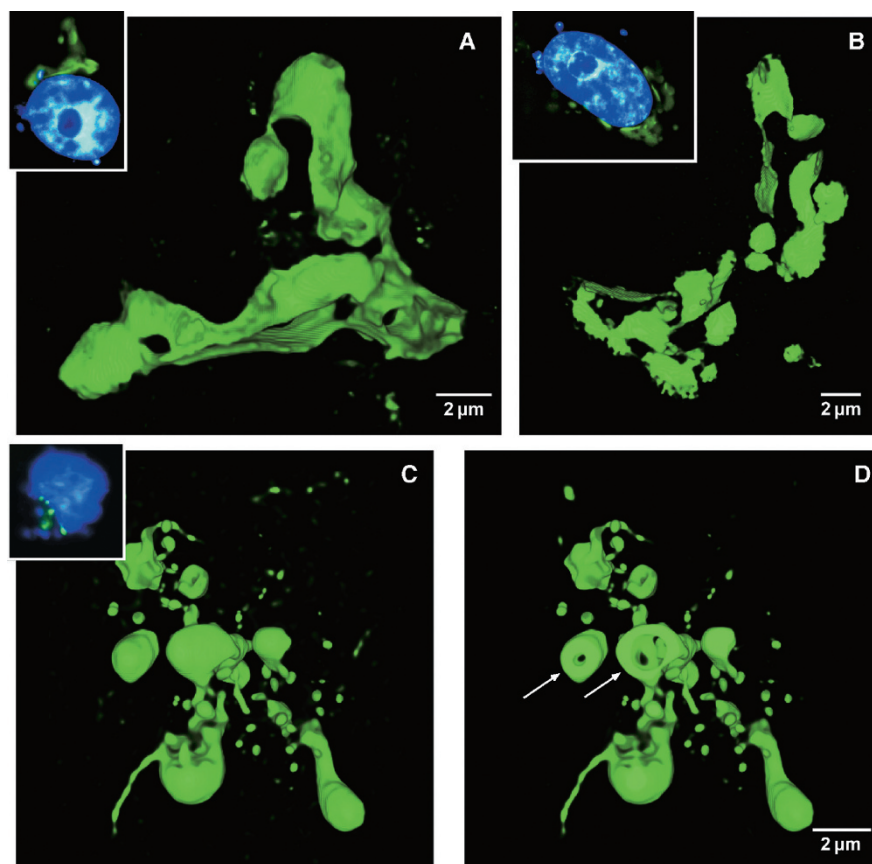


FIGURE 30.4. Golgi apparatus as obtained through 4Pi confocal imaging of GalT-EGFP (A) and 2-OST-EGFP (B) in living *Vero* cells at ~ 100 nm axial resolution. The insets show epi-fluorescence images to correlate the Golgi with the nucleus. The Hoechst staining of the nucleus indicates that the cells were in interphase. This was not the case for the GalT-EGFP transfected cell in (C). Though this cell is apoptotic, the data clearly pinpoint the ability of the 4Pi microscope to resolve small structures like the cavities, which become apparent when the upper caps of the balloon-like structures are removed (D).

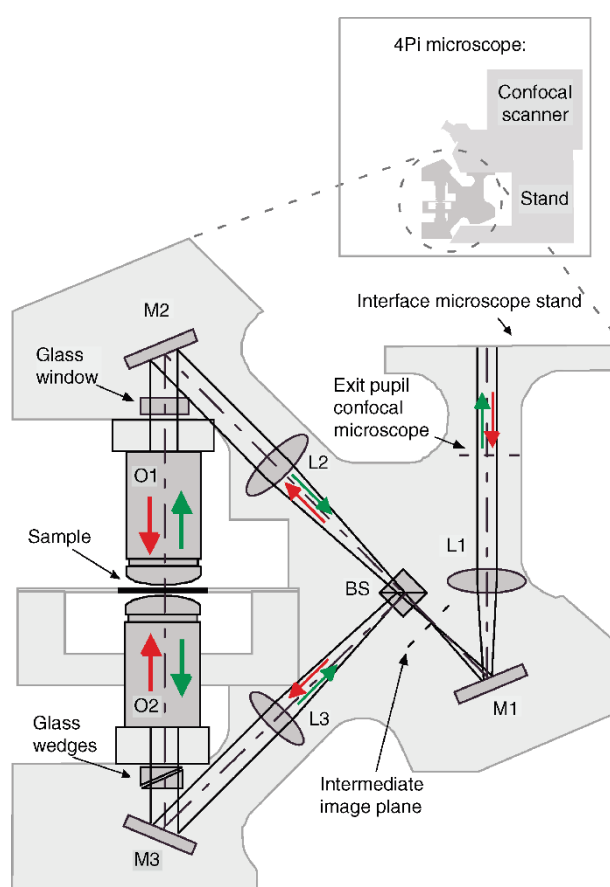


FIGURE 30.5. Sketch of the Leica TCS 4Pi. Excitation light originating from the microscope stand is divided by the beam-splitter (BS) and focused onto the same spot by the opposing objective lenses O1 and O2. The lenses L1, L2, L3 and the mirrors M1, M2, M3 form the intermediate optical system, ensuring that the two scanning pivotal points coincide with the entrance pupils of the two objective lenses. Fluorescence is collected by both lenses, recombined at BS and directed towards the microscope stand. Dispersion compensation over a large wavelength range is ensured by movable optical wedges in the lower interferometric arm whose thickness is compensated by a glass window in the upper arm. Scanning is performed by a standard confocal microscope unit.

30.5). For 2PE, the beam of a mode-locked Ti:Sa laser is coupled into the confocal microscope and directed towards the 4Pi unit, where it is split into two beams of equal intensity by the beam-splitter BS and focused into the sample by the objective lenses O1 and O2. To maintain the beam-scanning capabilities of the confocal microscope, the former exit pupil of the confocal microscope is imaged into the entrance pupils of the objective lenses by the telescope optics L1, L2, and L3.

In order to minimize the effects of thermal fluctuations, the 4Pi unit is designed symmetrically. The objective lenses are mounted on a mechanical translation stage and a piezo-mechanical translation stage, respectively. This allows the alignment of the two objective lenses along a common optic axis and the concurrence of their foci. The excitation beams can be aligned parallel to the optic axis of the objective lenses, as well as centric to the entrance pupil of the lenses, by adjusting the mirrors M1, M2, M3, and the beam-splitter. The mirrors M2, mounted on a mechanical translation stage, and M3, on a piezo-translation stage, also allow the adjustment and control of the balance of the interferometric arms.

Lateral scanning is performed by the usual fast tilting of the beam in the pupil of the lenses. Axial scanning is accomplished by translating the sample along the optic axis with a piezo scanning stage. The optical design of the 4Pi unit ensures invariance of the 4Pi-PSF across a field of 50 μm diameter. The sample stage is horizontally oriented, as in the case of standard confocal microscopes. In fact, the typical scanning conditions of a standard confocal scanner are retained.

The fluorescence is collected with both objective lenses, merged at the beam-splitter and partially reflected backwards to the confocal microscope, producing a Type C, 4Pi microscope. In contrast to the Type A mode, the beam-splitter divides the fluorescence light into a constructively interfering part that is directed towards the detection pinhole (corresponding to a detection PSF consisting of a central peak and symmetrically arranged sidelobes) and a destructive part (featuring a central minimum) emitted at the fourth side of the beam-splitter cube. The light emerging from a homogeneously fluorescing sample is in this way divided into two beams of half the available brightness. This loss is offset by the doubled detection efficiency achieved by the use of two objective lenses. However, the combination of constructive 4Pi excitation and constructive detection sharpens the central peak of the effective PSF and lowers the sidelobes.

Even though the same optical components are used in both interferometric arms of the 4Pi unit, the relevant thickness of the glass components is not exactly the same. In a Type A 4Pi microscopy, the resulting optical path difference can be adjusted by moving one of the mirrors along the optic axis, thereby changing the length of the air spacing. This is not possible in Type C 4Pi microscopy. Due to the different dispersion in the glasses and in air, dispersion compensation is necessary to balance the arms for multiple wavelengths. This is implemented by a pair of adjustable glass wedges in one of the interferometric arms. The mean thickness of the wedge-pair is precompensated in the other arm by a glass window of the average thickness of the wedges. The effective dispersion can be tuned by displacing the wedges against each other.

Resolution

The experimentally achievable resolution and sidelobe heights of 4Pi microscopy are shown in Figure 30.6 by measured z -responses of the Leica TCS 4Pi for water-immersion lenses (PL APO 63 $\times$ /1.20 W CORR, Leica Microsystems, Wetzlar, Germany) and glycerol-immersion lenses (HCX PL APO 100 $\times$ /1.35 GLYC CORR, Leica Microsystems, Wetzlar, Germany).

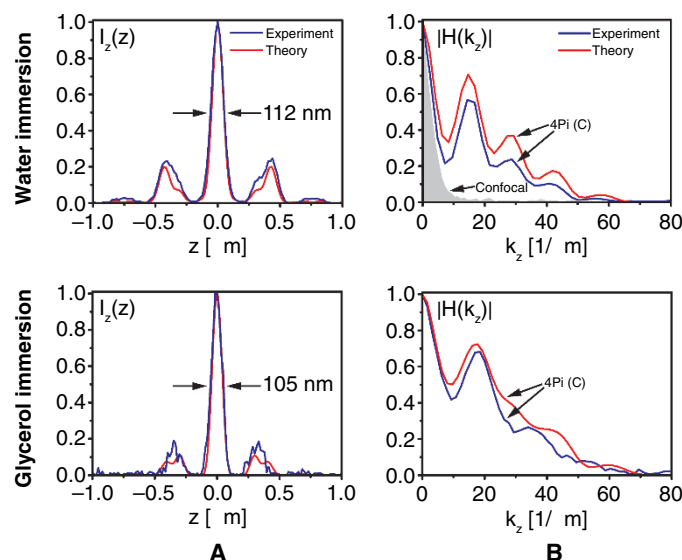


FIGURE 30.6. Experimental z -responses for water and glycerol-immersion lenses (PL APO 63 $\times$ /1.20 W CORR and HCX PL APO 100 $\times$ /1.35 GLYC CORR, Leica Microsystems, Wetzlar, Germany). The measurement for the water-immersion lenses was carried out with a fluorescent layer consisting of Oregon green dye molecules excited with laser light at a wavelength of 880 nm. The z -response of the glycerol-immersion lenses was obtained with a polydiacetylene layer excited at a wavelength of 840 nm. The central wavelength of the emission light is 565 nm in the case of the polydiacetylene layer, while it is 526 nm for the Oregon green layer. The pinhole diameter was set to the size of the backprojected Airy disk at the detection wavelengths used. The z -response of the water-immersion lens was recorded with an avalanche photodiode (APD), which results in a higher signal-to-noise ratio compared to the measured z -response of the glycerol-immersion lens recorded with a photomultiplier. For comparison, theoretical calculations are shown. To account for apodization at the periphery of the aperture, theoretical NAs of 1.15 and 1.30 were taken for the water- and the glycerol-immersion lens, respectively. The first minima in the OTF measured with the glycerol-immersion lens exhibits a value of 0.4. As a result of the higher sidelobes of the z -response, the value of the OTF measured with the water-immersion lens decreases to 0.22 at the first minima.

For both measurements, a detection pinhole diameter corresponding to the size of the backprojected Airy disk of the fluorescent light was chosen. A comparison of the measured axial responses with the theoretical ones (Fig. 30.6) shows remarkable agreement. The full-width at half-maximum (FWHM) of the z -response was measured as 105 nm and 112 nm for the glycerol- and water-immersion lens, respectively. In Type C 4Pi microscopy, the height and shape of the sidelobes are determined by the ratio of the excitation and the detection wavelength. The z -response of the glycerol lens features lower sidelobes in this experiment because of the slightly more suitable wavelength ratio, but also because the glycerol-immersion lenses feature an aperture angle that is larger by 4° than the water-immersion counterparts.

The OTF of the Type C 4Pi microscope along the reciprocal optic axis was calculated from the theoretical and experimental z -responses by Fourier transformation. It is completely contiguous with low intraband modulation. In fact, Type C 4Pi shows the best-filled OTF support reported with these types of microscopes (Nagorni and Hell, 2001). This well-filled OTF makes it suitable for routine operation. The largest transmitted object frequencies range between 45 μm^{-1} and 50 μm^{-1} . This corresponds to a 7-fold enlargement of the OTF compared to the confocal microscope (7 μm^{-1}).

Type C 4Pi Imaging in Living Cells

The suitability of Type C 4Pi microscopy in living cells was demonstrated by recording 3D images of the mitochondrial compartment in yeast cells (Gugel *et al.*, 2004). The mitochondrial matrix of *Saccharomyces cerevisiae* was labeled with green fluorescent protein (GFP). The cells were embedded in PBS with 1% low melting agarose and mounted between two coverslips. Apart from immobilizing the yeast cells, the agarose slightly increases the refractive index of the aqueous medium from 1.33 to 1.34. This reduces the effect of refractive index changes in the sample. The slight index mismatch between the medium and the immersion liquid induces a linear phase shift, which was actively compensated. The phase of the PSF in the stack was monitored with a fluorescent layer placed on one of the coverslips. The phase variation of the PSF throughout the whole stack was found to be less than $\pi/6$. Therefore, aberrations introduced by the biological sample were negligible in this case. The measurements were performed in a bidirectional scanning mode with a line frequency of 400 Hz. Due to the fast beam-scanning system, imaging speed is only determined by the sensitivity of the scanner. A typical mitochondrial stack of $6.25 \times 6.25 \times 4.5 \mu\text{m}^3$ ($x \times y \times z$) in size was recorded in 1.5 min. During this time span, the mitochondria did not change significantly. The wavelength of the excitation light was 880 nm.

The recorded data stacks were deconvolved with theoretical PSFs calculated according to the parameters used in the experiment, that is, a detection pinhole diameter corresponding to the size of the backprojected Airy disk of the light and an emission wavelength of 510 nm. The sidelobes obtained by recording the yeast mitochondria are of the same height as the sidelobes measured for the fluorescent layer, that is, below 30% of the main peak intensity. Figure 30.7 shows a typical surface-rendered reconstruction of a recorded stack. The data set was restored with nonlinear image processing using the Richardson–Lucy algorithm [Fig. 30.7(A)] as was applied for Type A 4Pi images in other publications (Nagorni and Hell, 2001). The good agreement of the measured axial response with the theory and the low sidelobes of the Type C 4Pi PSF enabled the recorded data stack to be linearly deconvolved. The linear filtering with a Wiener filter and an

adapted theoretical OTF not only removed the sidelobes but also increased the axial resolution to slightly better than 80 nm. Figure 30.7(B) illustrates the surface-rendered data set of the same mitochondrial compartment, but this time attained through linear image restoration. The similarity of the results obtained with linear filtering and nonlinear deconvolution demonstrates the applicability of linear filtering in Type C 4Pi microscopy.

SUMMARY AND OUTLOOK

With the latest instruments, MMM-4Pi and Leica TCS 4Pi, 4Pi microscopy has evolved from a laboratory imaging setup to an instrument for super-resolved 3D imaging of cells. Multiple images of the Golgi apparatus in living *Vero* cells (Egner *et al.*, 2004) as well as extensive studies of the topology of the mitochondrial network in live yeast cells (Egner *et al.*, 2002b) have exhibited reliable everyday operation. 4Pi microscopy has been shown to work with oil-immersion as well as with water- and glycerol-immersion lenses and can thus be applied under many biological imaging conditions for individual cells. However, at the present state of development, the allowable sample thickness is limited by two factors. First, the free working distance of the high NA lenses used sets an upper limit to the maximum distance between the two coverslips (typically ~80–200 μm). Second, thick strongly scattering specimens cause wavefront aberrations compromising the 4Pi interference. While for single cell layers 4Pi microscopy has been shown to work reliably, the applicability of 4Pi microscopy to somewhat thicker samples is left to be solved in the future. The usage of adaptive optics (Booth *et al.*, 2002) may be very helpful in that regard.

It is not absolutely necessary to mount the specimen between two coverslips: the upper lens can be a dipping lens. Combined with a standard water-immersion lens of the same NA and magnification in the lower arm, the Leica TCS 4Pi can be used with only one coverslip. This facilitates chemical, thermal, and also limited mechanical stimulation of the cells. Chambers have been developed allowing the buffered medium to be exchanged (Egner *et al.*, 2004), thus enabling the investigation of time-dependent changes as well as the effect of certain stimuli over many hours.

In conclusion, with the current 4Pi microscopes it is possible to record 3D data stacks from living cells in typically 1 to 20 min with an axial resolution of better than 100 nm. Strictly speaking, the lateral resolution does not change, but the significantly improved optical sectioning leads to an enhanced clarity of the details located in the focal plane. The MMM version of 4Pi microscopy has the potential of imaging a whole cell in less than a minute. By combining coherent excitation and detection, the Type C mode in the Leica TCS 4Pi allows imaging with a state-of-the-art confocal scanner featuring a confocal sectioning capability of ~80 nm.

A further increase in resolution can be achieved by merging 4Pi microscopy with other independent, resolution-enhancing techniques. Taking into account that the axial resolution is now slightly better than its transverse counterpart, rotating the sample (Bradl *et al.*, 1992; Heintzmann and Cremer, 2002) may allow a tomographic version of 4Pi microscopy which would further increase the 3D resolution optically. The combination of 4Pi and STED microscopy has already been demonstrated (Dyba and Hell, 2002; Dyba *et al.*, 2003) to attain an axial resolution of 33 nm. Being based on a reversible saturable transition (RESOLFT) (Hell, 2004; Hell *et al.*, 2004), STED has no inherent conceptual resolution limit. The limits are rather given by the limited performance

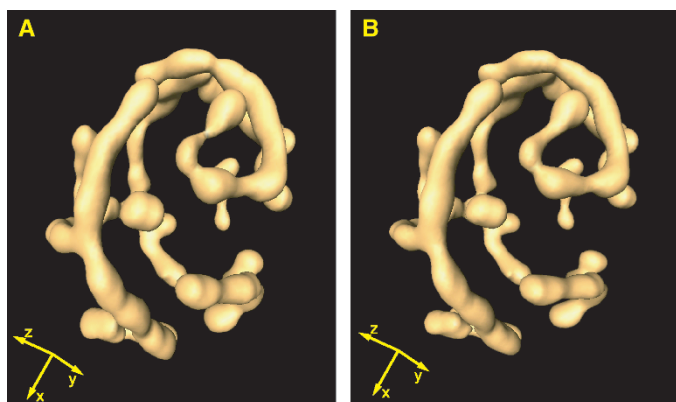


FIGURE 30.7. Mitochondrial network of a living yeast cell recorded with the Leica TCS 4Pi. The 3D image displayed was obtained after nonlinear (A) and linear (B) deconvolution and subsequent surface rendering of the raw 3D data. Note the good agreement of the two images indicating the efficiency of the mathematically simpler and arguably more reliable linear deconvolution. The mitochondrial matrix was labeled with EGFP. The diameter of the mitochondrial network shown is ~5 μm .

of the fluorophores in terms of emission cycles, quantum efficiency, and photobleaching. For living cells, this demand will be increased due to rapid movements of proteins and organelles within the sample. If these issues can be satisfactorily addressed in the future, the synergistic combination of STED or another RESOLFT mechanism is likely to provide true optical resolution of <10nm along the optic axis. At the same time, an increase in image acquisition speed through more sensitive and parallelized detection is expected.

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APPENDIX

I⁵M-OTF

Utilizing Köhler illumination with a lamp, I⁵M applies single-photon, plane standing-wave excitation. However, the fluorescence detection is as in a Type C 4Pi microscope: the spherical wavefronts of fluorescent light emerging at the sample are combined by

interference at the detector which is a CCD camera. The OTF of the I⁵M is much more structured than that of the 2PE Type A and Type C 4Pi microscopes (see Fig. 30.A1) and therefore I⁵M transfers data at some of these extended spatial frequencies only weakly. To visualize these components in the I⁵M-OTF, the inset of Figure 30.A1 displays the same OTF after normalization to 10% of its maximum value (Bewersdorf *et al.*, 2005). If the weakly transferred frequencies (at or even below the noise level) fall

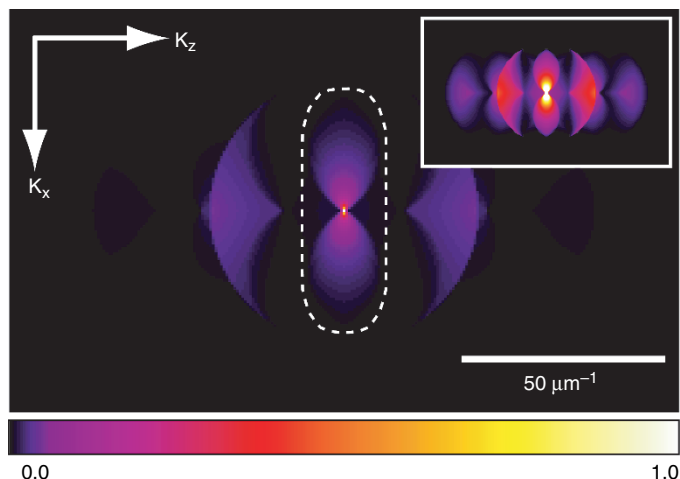


FIGURE 30.A1. OTF of the I³M. The inset shows the same OTF normalized to 10% of the maximum to highlight the weak regions of the OTF. The white dashed line shows the support of a standard confocal fluorescence microscope.

within the region of support of the OTF, periodic artifacts arise in the image. The white dashed line in Figure 30.A1 displays the support of the OTF of a normal confocal microscope. For the I³M-OTF calculations, a fluorescence wavelength of 510 nm, a single-photon excitation wavelength at 480 nm, and a 1.20 NA water-immersion lens were assumed. The height of the singularity in the center corresponds to an imaging size of $\sim 10 \mu\text{m}^3$.

Additional Information from the 4Pi-PSF

The sharper spot of the 4Pi microscope can be used to increase the localization accuracy of point-like objects in tracking experiments, or to enhance the measurement of distances between two known

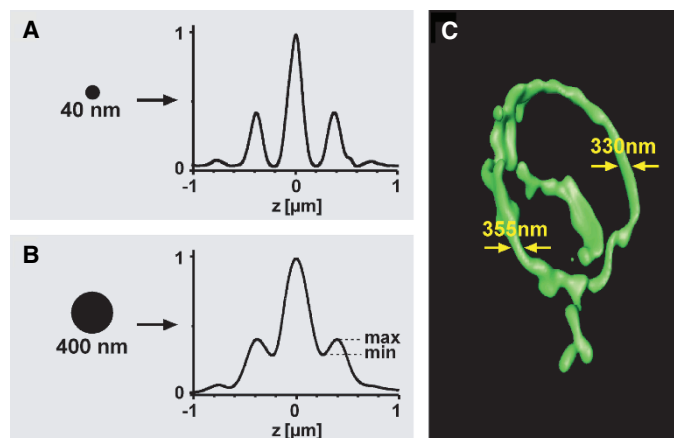


FIGURE 30.A2. Measuring diameters in the subresolution range with 4Pi microscopy. (A) and (B) show the intensity profiles through a laterally oriented rod with a diameter of 40 and 400 nm, respectively. The depth of the minima of the profiles is correlated to the thickness of the rod. By measuring the depth of the minima, the thickness of rod-like objects such as the yeast mitochondria can be derived (C).

objects. Likewise, knowledge about the symmetry of the feature of interest can be used to determine its center of mass and, therefore, its position. The precision of the position measurement is not challenged by diffraction and may far exceed the resolution (Gelles *et al.*, 1988; Bornfleth *et al.*, 1998; Lacoste *et al.*, 2000; Thompson *et al.*, 2002). The precision of this method scales, however, with the resolution and thus benefits from the narrow 4Pi focus. In fact, localization accuracies below 1 nm have been demonstrated with 4Pi microscopy (Schmidt *et al.*, 2000). Additionally, the 4Pi-PSF allows one to measure object thicknesses down to 60 nm. Figure 30.A2 illustrates the determination of the diameter of mitochondria in a yeast cell. In this example, it was possible to measure the average diameter with an accuracy of $\sim 5 \text{ nm}$ (Egner *et al.*, 2002b).