Axial Resolution Enhancement by 4Pi Confocal Fluorescence Microscopy with Two-Photon Excitation

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Abstract Confocal fluorescence microscopy and two-photon microscopy have become important techniques for the three-dimensional imaging of intact cells. Their lateral resolution is about 200–300 nm for visible light, whereas their axial resolution is significantly worse. By superimposing the spherical wave fronts from two opposing objective lenses in a coherent fashion in 4Pi microscopy, the axial resolution is greatly improved to ~100 nm. In combination with specific tagging of proteins or other cellular structures, 4Pi microscopy enables a multitude of molecular interactions in cell biology to be studied. Here, we discuss the choice of appropriate fluorescent tags for dual-color 4Pi microscopy and present applications of this technique in cellular biophysics. We employ two-color fluorescence detection of actin and tubulin networks stained with fluorescent organic dyes; mitochondrial networks are imaged using the photoactivatable fluorescent protein EosFP. A further example concerns the interaction of nanoparticles with mammalian cells.

Keywords 4Pi microscopy · Axial superresolution · Two-photon excitation · Fluorescent proteins · Photoactivatable proteins · Nanoparticle–cell interaction

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1 Introduction

Fluorescence microscopy has acquired a key role in life science research due to its ability to provide non-invasive three-dimensional (3D) imaging of cellular structures and processes, including living cells and tissues. In recent years, significant advances have been made in improving the spatial resolution of fluorescence imaging [1]. A variety of concepts have been introduced, including coherent wave front superposition in 4Pi [2, 3] and I5M microscopy [4, 5], reversible saturable optically linear fluorescence transitions [6–9] and, most recently, stochastic detection of individual, switchable fluorophores in photoactivated localization microscopy (PALM) [10], fluorescence photoactivation localization microscopy [11], stochastic optical reconstruction microscopy [12], and PALM with independently running acquisition [13]. With some of these advanced schemes, image acquisition with a lateral (x/y) resolution in the range of 10–20 nm has been demonstrated.

For imaging subcellular territories in all three spatial dimensions, confocal or multiphoton microscopy is employed for optical sectioning along the axial (z) direction. With these methods, a typical axial resolution of ~ 500 nm can be achieved, which is much lower than in the lateral directions. 4Pi confocal microscopy is a technique in which the axial resolution is enhanced a factor of 5–7 by a superposition of two coherent wave fronts emanating from opposing objective lenses in a confocal microscope [14–17]. 4Pi microscopes are classified as type A, B, or C, depending on the coherent superposition of the exciting light, emitted light, or both, respectively. An instrument of type A has recently become commercially available (Leica TCS 4Pi). The interference of the two excitation wave fronts generates an interference pattern along the axial direction, which consists of a narrow central maximum with side lobes above and below the focal plane. The side lobes, which result from the incomplete solid angle of the interfering waves, can be suppressed by two-photon excitation (TPE) and confocal detection to less than $\sim 30\%$ of the main peak. Weak side lobes can be removed by deconvolution so as to yield an artifact-free 3D reconstruction.

Equally important as the microscopy hardware is the availability of powerful fluorescent markers with which the specimens can be stained. Genetically encoded fluorescent marker proteins (FPs) are of particular interest in this context, as these autofluorescent proteins are produced by the cells and tissues themselves and do not require additional staining procedures [18–22]. Most exciting is the recent advent of photoactivatable FPs, which can be switched between states of different color or intensity of fluorescence emission by specific irradiation [23–30]. Targeted photoactivation within a small volume (<0.1 femtoliter) can be performed by two-photon activation (TPA), as demonstrated for the photoactivatable proteins PA-GFP [31], KikGR [32], and EosFP [33, 34], allowing for in vivo tracking of the optically marked proteins. We have recently characterized the TPA and TPE properties of EosFP [33]. These parameters are crucial for the simultaneous 3D imaging of photoactivated and nonactivated regions by 4Pi microscopy.

In most cellular imaging applications, at least two spectrally distinguishable fluorescent labels are used simultaneously to reveal protein–protein interactions or localization of proteins in relation to cellular compartments. Dual-color imaging is much more challenging in TPE, which is employed in 4Pi microscopy, than in conventional fluorescence microscopy with one-photon excitation, because pairs of fluorescent labels are needed, where both dye molecules can be excited with the same laser wavelength but emit at markedly different wavelengths. A variety of TPE spectra of fluorescent dyes were reported over the years [35–38]. A careful study of these publications reveals some inconsistencies, which may arise from the different temporal structure of the laser excitation used [39], or from different

experimental environments. Therefore, we prefer to study the TPE properties of promising dyes for these applications directly in our experimental setup.

In the current study, we address the problem of marker dye selection for dual-color 4Pi microscopy. Moreover, we present cellular applications with different fluorescent labels, using conventional staining and genetic encoding of the labels.

2 Materials and Methods

2.1 4Pi Confocal Microscopy

4Pi images were acquired with a commercial 4Pi microscope with TPE (Leica TCS 4Pi, Leica Microsystems, Mannheim, Germany), using a matched pair of opposing objective lenses (HCX PL APO 100 $\times$ /1.35 GLYC CORR CS, Leica Microsystems) to achieve a coherent superposition of two exciting wave fronts (4Pi type A). For TPE, the picosecond Ti:Sapphire laser (Mai Tai, Newport Spectra-Physics, Mountain View, CA, USA) was tuned to a suitable excitation wavelength between 720 and 980 nm. The specific excitation wavelengths and spectral ranges of detection are given in the figure captions associated with the individual applications. 4Pi images were recorded as *xz*-scans, which are stacked in the *y* direction for 3D imaging using a typical voxel size of 15 $\times$ 15 $\times$ 30 nm for detailed images and voxels up to three times larger for overview images. A pinhole size of 1 Airy unit was usually used, and avalanche photodiode (APD) detectors were employed for increased sensitivity. For TPA of EosFP, a particular spot within the cell was illuminated with light of 810 nm for 20 s, with an excitation power of 80 μ W at the sample. Here, the lower objective was blocked to select a smooth, approximately 3D Gaussian volume instead of the complicated pattern produced by the interference of two wave fronts.

Images were processed with the Inspector software package [40]. Blurring was introduced in accordance with the sampling theorem, corrections were applied for background and crosstalk, and a one-dimensional linear three-point deconvolution was performed to remove side lobe artifacts without introducing any resolution enhancement. After rescaling, overlays were generated from the images of the red and the green channels. Image stacks were volume rendered using the program Voxx [41].

2.2 Two-Photon Excitation Spectroscopy

Relative two-photon action spectra were acquired using 100- μ M solutions of Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA) or Texas Red (Invitrogen) dissolved in a mixture of phosphate buffered saline (PBS; Gibco, Karlsruhe, Germany) and glycerol (13%/87% by volume). The spectra were adjusted for equal incident photon flux densities, for the spectral dependence of the diffraction-limited spot size, and for the measured transmission of the excitation path. Furthermore, the spectral dependence of the volume size was corrected in order to take the different number of excited molecules into account [38].

2.3 Sample Preparation

Escherichia coli membranes were stained by incubating a turbid suspension of *E. coli* with a 40- μ M buffer solution of the membrane stain DiI (Invitrogen) for 10 min. Subsequently, the samples were washed three times by centrifugation, removal of the supernatant, and

resuspension in PBS. The cell suspension was applied to quartz coverslips coated with poly-L-lysine (Sigma-Aldrich, St. Louis, MO, USA). The cells were allowed to adhere for 10 min before the coverslips were washed with buffer.

HeLa cells (ATCC CCL 2) were cultured at a density of 10^5 cells per cm^2 on circular quartz coverslips (Leica), coated with 50 μg of human fibronectin from plasma (Roche Diagnostics, Mannheim, Germany). For immunostaining, the cells were fixed after 16 h with 4% paraformaldehyde (PFA; Merck, Darmstadt, Germany) in phosphate buffered saline. PFA was inactivated with 50 mM ammonium chloride in PBS for 10 min. After permeabilization with 0.1% Triton X-100 in PBS for 10 min, nonspecific staining was blocked by incubating the cells in 0.2% fish skin gelatin (G7765, Sigma-Aldrich) in PBS for 30 min. Tubulin fibers were stained with an anti- α -tubulin antibody (1:2,000 dilution of mouse monoclonal IgG, T9026, Sigma-Aldrich) and a 1:1,000 dilution of a goat anti-mouse IgG-Alexa Fluor 488 (Invitrogen) together with 0.2 U/ μl phalloidin-Texas Red (Invitrogen) for actin staining. For photoconversion experiments with EosFP, the plated cells were transfected after 16 h with 2 μg of the expression vector pcDNA3-mitochondrial targeting (mt)-EosFP using the FuGENE transfection reagent (Roche Diagnostics). In this vector, cDNA coding for EosFP is fused to cDNA coding for the mitochondrial targeting signal from subunit VIII of cytochrome c oxidase [27]. Cells were rinsed with PBS 24 h after transfection and fixed with 4% PFA for 10 min at room temperature.

Mesenchymal stem cells for nanoparticle penetration experiments were plated on quartz coverslips and incubated for 24 h with yellow-green beads of 100 nm diameter (Fluo-Spheres, carboxylate modified, Invitrogen). For membrane staining, a concentration of 10 mM DiD (Invitrogen) was added for 2 min. After a single washing step, the cells were fixed with 4% PFA for 10 min and washed again.

For all experiments, sandwiches of two quartz coverslips were assembled with PBS/glycerol (13%/87% by volume) as a mounting medium. All experiments were carried out in a temperature-controlled laboratory at 22°C.

3 Results and Discussion

3.1 Axial Superresolution in 4Pi Microscopy Applications

4Pi microscopy of type A utilizes the interference of two coherent laser beams that are focused on the same point within a fluorescently labeled sample by means of two opposing objective lenses (Fig. 1). This interference creates a sharp central peak in the point spread function (PSF) in the axial direction that is accompanied by side lobes. The PSF can be determined by imaging objects smaller than the axial resolution, for example, fluorescent particles of <100 nm diameter. From the PSF, the relative height of the side lobes and their distance from the center peak can be extracted. These parameters are utilized in a simple three-point deconvolution algorithm which only assumes identical shapes of the side lobes and the main peak to remove side lobe artifacts. Consequently, it does not involve deconvolution with a true PSF for resolution enhancement and, thus, minimizes the risk of deconvolution artifacts.

To demonstrate axial superresolution and deconvolution in 4Pi confocal microscopy on a biological object, we immobilized *E. coli* bacteria and resolved their outer membranes, which were stained with the carbocyanine dye DiI. In Fig. 2, three bacteria in different orientations are shown in the xz plane. Figure 2a shows the 4Pi raw image with a profile

Fig. 1 Optical geometry in 4Pi microscopy. The exciting near-infrared light (*black arrows*) is split symmetrically by a beam splitter cube and coherently superimposed within the sample by two opposing objective lenses. Fluorescence emission is collected by the same objectives and guided back (*gray arrows*) into the detection path of the confocal microscope. An example of an instrumental PSF after confocal detection is shown on the *left*

along the indicated line below the image. The narrow main peaks (full width at half maximum ~ 140 nm) representing the membrane are accompanied by two slightly asymmetric side lobes each. After deconvolution (Fig. 2b), the sidelobes are almost completely removed; a sharp image of the membrane remains. The resolution is strongly enhanced in the axial direction as compared to a confocal recording of the same cells (Fig. 2c), whereas no difference in resolution can be observed in the x -direction. The incomplete removal of

Fig. 2 Fluorescence images of three *E. coli* bacteria with DiI-stained membranes. **a** 4Pi raw image in the xz plane after smoothing and background correction exhibiting ghost images *above* and *below* due to the side lobes of the PSF. **b** The same 4Pi image after linear three-point deconvolution. **c** Confocal image of the same objects using only the bottom objective. *Below*, we have plotted intensity profiles along the *lines* indicated in the corresponding images. Images were taken with an excitation wavelength of 758 nm and a detection band of 535–585 nm

secondary interference maxima is mainly caused by imperfect optical conditions within the sample. In such a case, the images can be further improved by deconvolution with a spatially varying PSF [42]. Alternatively, axial phase shifts can be actively compensated during data acquisition, as was recently introduced by Leica Microsystems. Further improvements in 4Pi microscopy are expected from oil immersion objective lenses with higher numerical apertures. Their larger aperture angles give rise to reduced sidelobes so that 4Pi microscopy with one-photon excitation may become feasible [43].

3.2 Fluorescent Markers for Dual-Color 4Pi Imaging

Good fluorescence markers for 4Pi microscopy should have large two-photon excitation cross sections and be resistant to multiphoton bleaching. For dual-color 4Pi imaging, two marker dyes are required that can be efficiently excited by two-photon absorption with the same laser wavelength but differ markedly in their emission spectra. Furthermore, a high quantum yield of the marker dyes under 4Pi imaging conditions is advantageous. All these properties depend on the solvent and even the choice of the laser source may change the experimental two-photon excitability. Therefore, we have characterized the dyes in our standard mounting medium for 4Pi imaging with glycerol objectives (87% glycerol, 13% PBS), using the 4Pi microscope itself for the measurements. Because the APD detectors register the absolute number of photons, different dyes can be compared by their relative two-photon action spectra, which represent the experimental brightness of both fluorophores in 4Pi microscopy applications for equal incident photon flux densities. By applying corrections for the individual spectral detection efficiencies and quantum yields in the solvent mixture, absolute two-photon excitation cross sections can be obtained by calibration against a reference dye [33, 38].

Figure 3 shows the two-photon action spectra of the fluorescent dyes Alexa Fluor 488 and Texas Red determined as described. Texas Red shows two prominent excitation bands around 740 and 890 nm, whereas Alexa Fluor 488 has almost ten times less two-photon excited fluorescence in its main band around 760 nm, which appears as a double-humped peak. The two-photon excitation spectra resemble those previously published for Texas Red [44] and Alexa Fluor 488 [37] in aqueous solvent. Thus, we conclude that the glycerol

Fig. 3 Comparison of two-photon excitation action spectra of Alexa Fluor 488 and Texas Red. The two-photon excitation efficiencies of both dyes are shown on the same arbitrary scale, which represents the detected fluorescence signal at the respective excitation wavelengths. The values for Alexa Fluor 488 are magnified by a factor of two for better visibility

environment does not strongly affect the spectral shape. From the two-photon action spectra, the optimal range of excitation wavelengths for dual-color 4Pi microscopy can be extracted. However, multiphoton bleaching often prohibits the use of certain excitation wavelengths. This is the case for Texas Red, which rapidly photobleaches upon TPE in the 740-nm band, in accordance with the observation that the quadratic power dependence of the fluorescence emission saturates already at low powers in this wavelength range. In an ongoing 4Pi microscopy study of neuronal synapses, we use an excitation wavelength of 800 nm to achieve similar fluorescence emission from the presynaptic and the postsynaptic regions, which are immunolabeled with Texas Red and Alexa Fluor 488, respectively.

3.3 Dual-Color 4Pi Microscopy of Biological Samples

Color multiplexing is frequently used in biological imaging applications to determine the mutual positions of two or more different proteins or structures. Different target proteins can be specifically labeled by binding of a specific antibody (immunolabeling). As an application example, we used the marker dyes Alexa Fluor 488 and Texas Red for simultaneous 4Pi imaging of the tubulin and the actin cytoskeletons within PFA-fixed HeLa cells (Fig. 4). With the proper choice of the excitation wavelength, which also depends on the individual staining efficiencies, similar fluorescence emission from both structures can be achieved, and both structures can be visualized simultaneously at high resolution.

Specific fluorescence tagging of proteins can also be achieved by fusing the DNA coding for a particular protein to the DNA coding for a fluorescent protein and then expressing the fusion construct in cells. Even more information can be gained by using photoactivatable FPs. These optical marker proteins allow the fluorescence emission intensity or color to be controlled by irradiation with light at specific wavelengths. Photoactivatable proteins are important tools for subdiffraction imaging [9–11, 13] and enable localized optical marking of subcellular regions and subsequent *in vivo* tracking of the marked proteins. In standard fluorescence microscopy with one-photon excitation, the optically marked region is limited by the size of the illuminating laser spot in the lateral dimensions, but there is no selective marking along the axial dimension. Confinement in all three spatial dimensions is only possible by using two-photon activation. In addition, 4Pi confocal microscopy can be used to achieve a high axial resolution of ~ 100 nm. Thus, the combination of two-photon

Fig. 4 Dual-color 4Pi image (after three-point deconvolution) of tubulin and actin networks in a PFA-fixed HeLa cell using Alexa Fluor 488 and Texas Red as marker dyes. The image was taken with 830-nm excitation and simultaneous detection in two spectral channels. **a** The green channel (500–550 nm) shows the microtubule network stained with mouse anti- α -tubulin and secondary antibodies labeled with Alexa Fluor 488. **b** The red channel (572–648 nm) shows the actin network stained with a phalloidin-Texas Red conjugate. **c** Overlay image of microtubule (light gray) and actin (dark gray) cytoskeletons

Fig. 5 Localized two-photon photoactivation of mt-EosFP in mitochondria. A 4Pi image of an EosFP-labeled mitochondrial network of a PFA-fixed HeLa cell is shown in form of a volume-rendered 3D reconstruction of a projection in the xz plane. Fluorescence was excited at 970 nm and detected in a green (500–530 nm) and a red (572–648 nm) spectral channel; emission into the latter channel is due to the two-photon converted regions (shown in *light gray*)

activation with high-resolution 4Pi microscopy is ideally suited for 3D tracking of protein subpopulations carrying a photoactivatable marker.

EosFP is an autofluorescent protein that can be switched from green to red emission by irradiation with ~ 400 -nm light, which causes a photoinduced backbone cleavage with a concomitant extension of the delocalized π electron system of the fluorophore [27, 30, 34]. We have used EosFP for a combination of 3D optical marking of subcellular regions and 4Pi confocal microscopy. To this end, EosFP was fused to a mitochondrial targeting signal and expressed in HeLa cells [33]. After two-photon conversion (810 nm) of a small subregion within the mitochondrial network of a HeLa cell, the photoconverted volume was resolved in a 3D reconstruction from a dual-color 4Pi image stack (970 nm). A projection of the image stack, in which the photoconverted region can be clearly identified, is shown in Fig. 5.

Fluorescent nanoparticles are alternative markers in fluorescence imaging which are becoming increasingly popular, owing to their superior brightness and photostability. Nanoparticles are also used in many industrial and medical applications, which raises the

Fig. 6 Cell penetration of nanoparticles. 4Pi two-photon microscopy image of a PFA-fixed mammalian cell after 24 h of incubation with yellow-green latex beads of 100 nm diameter. Images were taken with an excitation wavelength of 850 nm, recording the fluorescence of the latex beads in a green (500–550 nm) and the membrane stain DiD in a red (647–703 nm) spectral channel. All latex beads (shown in *light gray*) in proximity of the cell membrane were taken up by the cell

necessity of studying their interaction with cells to avoid potentially adverse effects on human health [45]. As yet, little is known about the detailed molecular mechanisms that govern nanoparticle uptake of cells as well as the consequences of nanoparticle incorporation on cell function, including differentiation and proliferation. 4Pi fluorescence imaging is a minimally invasive technique that allows the study of nanoparticle–cell interaction, uptake, and intracellular targeting with high spatial resolution. In combination with suitable dyes, simultaneous imaging of the cell membrane and nanoparticles is possible. We have used 4Pi fluorescence microscopy to study the uptake of fluorescently labeled latex particles of 100 nm diameter by membrane-stained mammalian cells. After 24 h of incubation, simultaneous two-photon excitation and imaging of the stained cell membrane and yellow-green labeled nanoparticles was performed (Fig. 6). The improved axial resolution of 4Pi microscopy allows internalized and noninternalized nanoparticles to clearly be discerned. Interestingly, latex particles (shown in light gray) were not observed in the vicinity of the cell membrane, suggesting that all particles in touch with the membrane were internalized by the cell.

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