Effective Axial Resolution in Single-photon 4Pi Microscopy

The feasibility of single-photon 4Pi-confocal microscopy is theoretically demonstrated. By inserting a pair of properly designed multi-ring phase-only pupil filters in the illumination path of a 4Pi microscope the height of the sidelobes of the point spread function is importantly reduced, so that there is not ambiguity in the 3D image. Then, an axial resolution up to four times higher than that of single-photon confocal microscope can be effectively achieved.

Introduction

Confocal scanning microscopy is an imaging technique whose main feature is its proverbial optical sectioning capacity [1]. Although confocal technique was specifically designed to improve the axial resolution, it is remarkable that due to the laws of diffraction the resolution in the direction of the optical axis is about three times poorer than its lateral counterpart. This difference between axial and lateral resolution leads to anisotropic images of three-dimensional (3D) specimens.

One attempt to reduce this anisotropy is to insert in the illumination arm of the confocal setup a pupil filter composed by two equal-area transparent rings [2]. With this technique it is possible reduce the axial extent of the point spread function (PSF) up to a factor 1.5. A very ingenious technique for reducing the axial extent of the PSF is to create a standing wave by the interference of two opposing wave-fronts, as made in the so-called 4Pi-confocal microscopy [3]. In the 4Pi-confocal microscope two opposing high-NA objectives are used for coherently illuminating and detecting the same point of a fluorescent specimen. The resulting PSF has an axial main peak that is about four times narrower than its confocal counterpart. However, the narrowing of the main peak is accompanied by a severe enlargement of the secondary axial sidelobes, which lead to ambiguity in the image.

Keywords

Confocal microscopy, Axial resolution, Apodization, Single-photon fluorescence

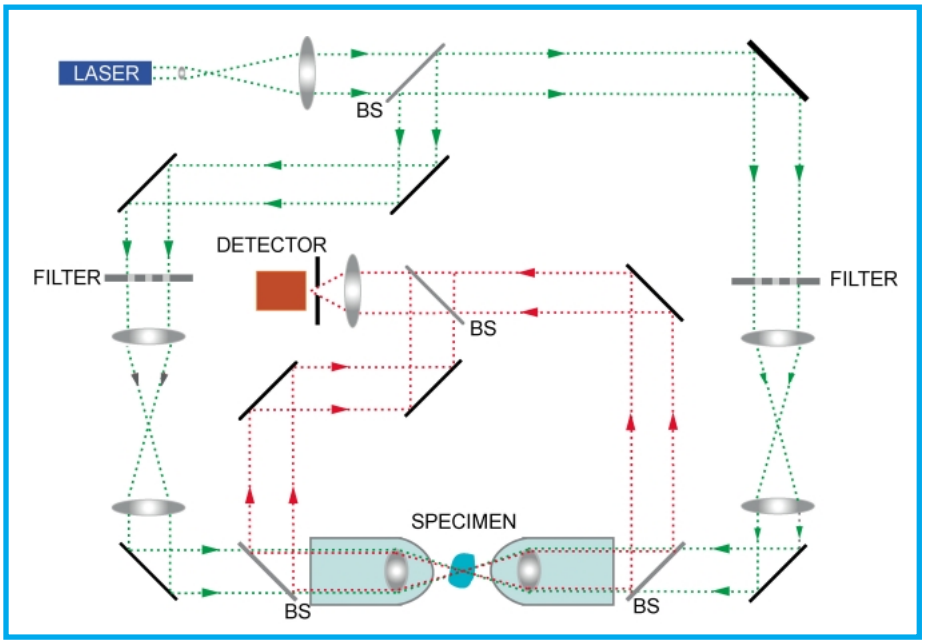

Fig. 1: Schematic lay out of a 4Pi(c)-confocal microscope. Relay lenses are used to focus the pupil filters into the back focal planes of the objectives.

To overcome this problem, it was proposed the use of two-photon excitation [4], which allow an important reduction of the height of the secondary axial lobes. Then data deconvolution techniques can be used to improve the quality of the image [5].

What we report here is that by using a pair properly designed phase-only pupil filters, the axial sidelobes of the PSF can be strongly downed. This implies that single-photon 4Pi-confocal microscopy can produce unambiguous 3D images, and then it makes not necessary to resort to multi-photon processes. The phase-filters are designed according to the axial form of the Toraldo's pupil-synthesis method [6,7], and are composed by seven annular transparent zones.

The 4Pi confocal principle

Consider a 4Pi(c)-confocal scanning microscope [3] like the one plotted in figure 1. The 3D specimen is illuminated by the standing wave generated by the interference between the two opposing, tightly focused waves proceeding from the high NA objectives. The light emitted by the fluorescent specimen is collected by the same objectives, and interfere at the pinhole plane. If we consider that the illuminating beam is linearly polarized, the 3D PSF of this imaging system is given by

$$I_{4Pi}(z,r,\varphi) = I_{ill}(r,z,\varphi)\, I_{det}(r,z) = \left| E(r,z,\varphi) + E(r,-z,-\varphi) \right|^2 \left| E(\varepsilon r,\varepsilon z) + E(\varepsilon r,-\varepsilon z) \right|^2 \quad (1)$$

where random polarization of fluorescent light has been assumed. The parameter $\varepsilon = \lambda_{exc} / \lambda^{fl}$ represents the ratio between the excitation and the fluorescence wavelength. In eq.(1) the function $E(r,z,\varphi)$ denotes the electric field in the

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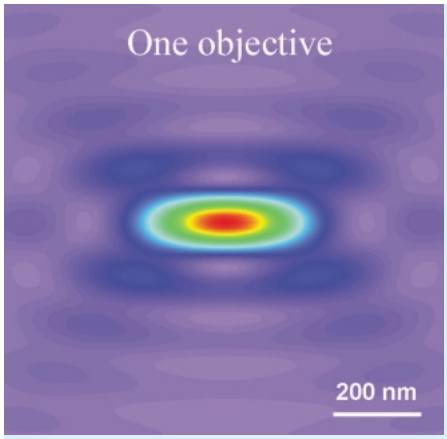

Fig. 2a

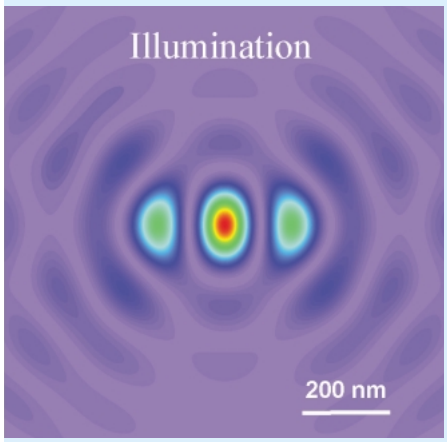

Fig. 2b

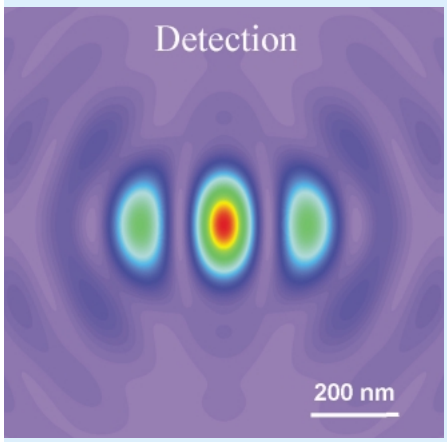

Fig. 2c

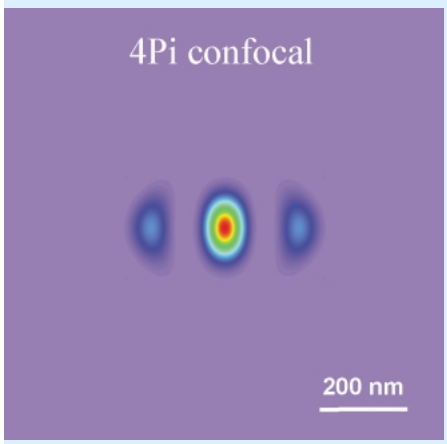

Fig. 2d

focal region of an aberration-free lens illuminated by a linearly-polarized plane wavefront. According to Richards and Wolf [8],

$$E(r,z,\varphi) = [I_0(r,z) + I_2(r,z)\cos 2\varphi]\,\mathbf{i} + I_2(r,z)\sin 2\varphi\,\mathbf{j} - 2iI_1(r,z)\cos\varphi\,\mathbf{k} \quad (2)$$

where $I_{0,1,2}$ are integrals over the aperture angle and φ stands for the angle between the polarization direction of the incident field and the observation meridian plane.

An example of 3D PSF is shown in figure 2, where we selected for the calculations $NA = 1.4$ ($n = 1.518$, $\alpha = 67.5$), $\lambda_{exc} = 350$ *nm* and $\varepsilon = 0.8$. Note from this figure that the PSF exhibits, along the axial direction, a very narrow central lobe, but high axial sidelobes that can produce artifacts in the 3D image.

The use of phase-shifting apodizers

To overcome the sidelobes problem we propose to place in the illumination path of a single-photon 4Pi-confocal microscope, a pair of properly-designed phase-only pupil filters. This produces a significant reduction of the axial-PSF sidelobes, and therefore makes it unnecessary the use of two-photon processes. The filters are inserted in the illumination path to avoid waste of fluorescence light.

Following the Toraldo's method [7] we have designed a pupil filter that consists of seven annular zones. The zones have no absorption, having each pair of neighboring zones opposite phases, see figure 3. The choice of phase-only filters with constant transmittance in the annular zones yields to a design that can be manufactured with relative ease. In figure 4 we show the, numerically evaluated, PSF of a 4Pi-confocal microscope in which two copies of the above phase filter are inserted in the illumination path. The parameters for the calculation were the same as the ones used in figure 2. Apart from strongly reducing the axial sidelobes, the use of Toraldo filters hardly affects to the transverse extent of the PSF.

To illustrate the utility of our proposal a numerical imaging experiment was performed. We designed an elaborated synthetic 3D object consisting of two concentric spherical fluorescence labeled shells, as shown in figure 5(a). The test object was designed to contain all the axial and transverse frequencies. The dark band in the object permits to clearly visualize the improvement in resolution along the different directions passing

Fig. 2: Numerically evaluated contour plots of the 3D intensity PSF in the meridian plane $\varphi = \pi/2$ corresponding to: (a) One objective; (b) Two opposing illumination objectives; (c) Two opposing collection objectives; and (d) The 4Pi single-photon confocal microscope. The parameters for the calculation were $NA = 1.4$, $\lambda_{exc} = 350$ *nm* and $\lambda_{fl} = 440$ *nm*

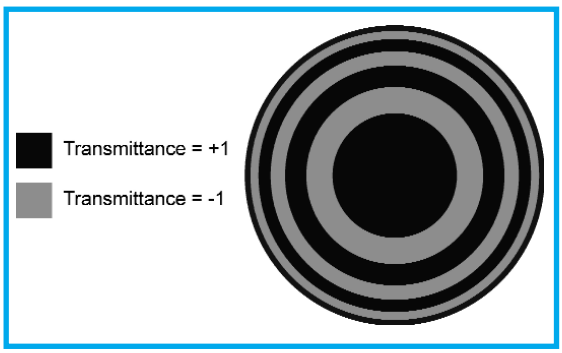

Fig. 3: The seven-zone Toraldo filter

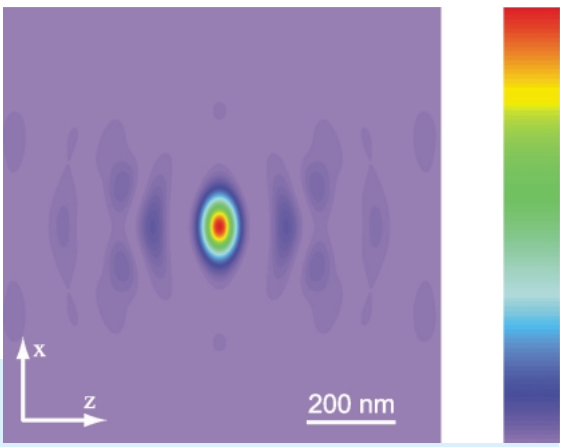

Fig. 4: Contour plot of the intensity PSF corresponding to the apodized 4Pi confocal microscope.

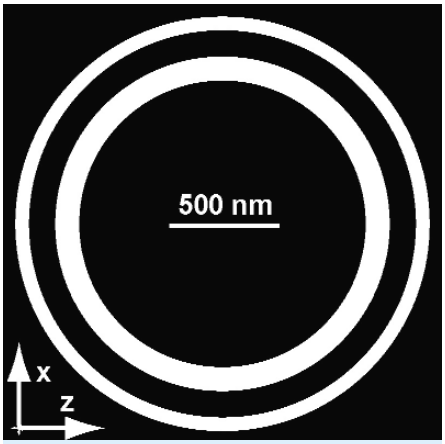

Fig. 5a

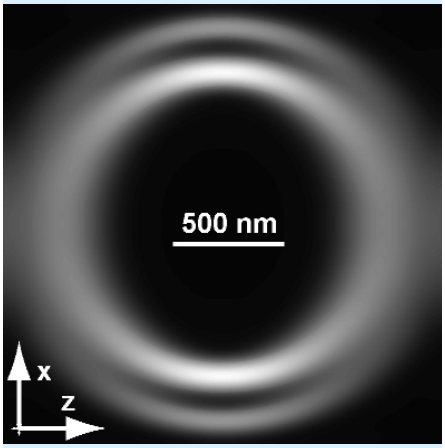

Fig. 5b

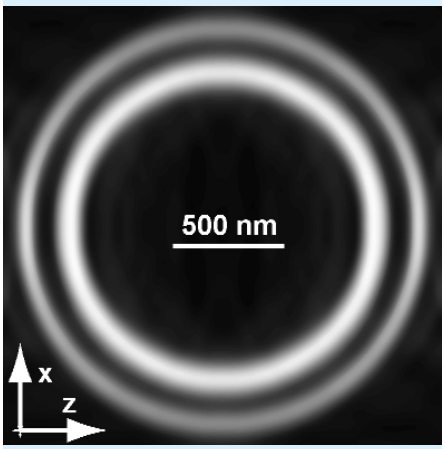

Fig. 5c

Fig. 5: Imaging of the synthetic 3D object: (a) Test object consisting of two concentric spherical fluorescence labeled shells; (b) Axial section (x,z) of the calculated mage for the case of the confocal microscope; (c) Same as (b) but for the case of the apodized 4Pi microscope.

through the focus. After convolution with the PSF of the 4Pi microscope (Fig. 4) and with the one corresponding to the confocal counterpart, we obtained the simulated images shown in figure 5(b-c). As can be noticed from the images the optical sectioning capacity of the apodized 4Pi is really superior to that of the confocal microscope.

Conclusions

The viability of single-photon 4Pi-confocal microscopy is theoretically demonstrated. By using multi-ring phase-only pupil filters in the illumination path of the microscope it is possible to down the axial sidelobes height to 7 % of the main peak. This allows the system to receive the full benefit in axial resolution from a main peak that is about four times sharper than that of a confocal microscope. The combination of the proposed technique with deconvolution techniques would make it unnecessary the use of multi-photon processes.

Acknowledgements

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