Determination of the unknown phase difference in 4Pi-confocal microscopy through the image intensity

Carlo Mar Blanca, Jörg Bewersdorf, Stefan W. Hell*

High Resolution Optical Microscopy Group, Max Planck Institute for Biophysical Chemistry, Am Fassberg 11, 37070 Göttingen, Germany

Received 27 November 2001; received in revised form 5 March 2002; accepted 1 April 2002

Abstract

We investigate the effect of the phase difference of the interfering wavefronts on the image intensity in 4Pi-confocal microscopy and thereby introduce a method to diminish the problem of the unknown phase difference in this microscope. An efficient implementation of our method utilizes binary amplitude filters reshaping the point-spread-function such that a significant disparity arises between the maximum fluorescence intensity in the constructive and destructive interference case. In the presence of planar and point-like features in the sample this property enables the system to unambiguously determine the mode of interference. © 2002 Elsevier Science B.V. All rights reserved.

PACS: 42.30.-d; 42.30.Va; 87.64

1. Introduction

Research in intracellular structures has spurred the development of microscopes that rely on the use of interfering wavefronts of opposing lenses for improved axial resolution. Examples include the 4Pi-confocal [1], the incoherent interferential illumination microscope (Î“M) [2,3], and the standing wave microscope [4]. The 4Pi-confocal and the Î“M have delivered 3D-resolution at the 100 nm scale [3,5–7], but an indispensable requirement of these methods is the determination of the exact phase difference $\phi$ between the interfering beams at the common focal point. The value of $\phi$ decides whether the effective point-spread-function (E-PSF) is constructive, featuring a sharp main maximum in the center, or destructive with a central naught. The precise knowledge of the E-PSF is essential, since the periodicity artifacts induced by the interference side-lobes usually necessitate image post-processing [8]. The E-PSF and hence the phase difference are obtained by imaging simple fluorescent structures representing isolated point-like particles. The transverse integral of the E-PSF, that is the $z$-response, is equally useful; it is obtained by axially scanning an isolated ultrathin layer [9]. In many
cases the specimen features itself point-like or sparse planar structures, but not always in the area to be imaged. In this case test-objects can be built-in by special sample preparation, such as coating of the coverglass with fluorescent monolayers [9], or dabbing the specimen with dyed microspheres. In general, the search for phase-indicative structures and the recording of the whole E-PSF or $z$-response can be cumbersome. Therefore, a simpler determination of the phase difference is required. In this paper we analyze the effect of the phase on the image intensity and experimentally demonstrate a novel phase-establishing method that is based on a simple measurement of the intensity.

2. Concept and theoretical results

The most obvious criterion for distinguishing constructive from destructive interference in a (two-photon excitation) 4Pi-confocal fluorescence microscope would be a difference in total image intensity. Such differences are indeed found but they are pronounced only for comparatively large aperture angles. This is demonstrated in Fig. 1, in which we studied the ratio between the integrated signal of the E-PSF for a two-photon excitation microscope as a function of the semi-aperture angle $\theta_{\text{max}}$

$$\Gamma = \frac{\int_{-\infty}^{\infty} I^{\phi=\pi}(z)dz}{\int_{-\infty}^{\infty} I^{\phi=0}(z)dz} < 1, \quad (1)$$

whereby

$$I^{\phi}(z) = C \int_{-\infty}^{\infty} [\tilde{E}_1(z, r, \varphi) + \tilde{E}_2(-z, r, \varphi)] \exp(i\phi)^{4}[h_{\text{det}}(z, r) \otimes p(r)]rdrd\varphi$$

$$= C \int_{-\infty}^{\infty} h_{\text{eff}}(r, z, \varphi)rdrd\varphi \quad (2)$$

denotes the $z$-response with the phase difference $\phi$ as parameter. $\tilde{E}_{1,2}(z, r, \varphi)$ are the counter propagating focal fields of the two spherical wavefronts [1], with $\varphi$ denoting the azimuthal angle. The expression $h_{\text{det}}(z, r) \otimes p(r)$ is the convolution of the detection-PSF with the detector pinhole opening. We further introduce

$$A = \max \frac{I^{\phi=\pi}(z)}{I^{\phi=0}(0)} < 1 \quad (3)$$

and

$$P = \max \frac{h_{\text{eff}}^{\phi=\pi}(z)}{h_{\text{eff}}^{\phi=0}} < 1. \quad (4)$$

$A$ gives the ratio between the maximum brightness of a thin layer when changing from constructive to destructive mode imaging and $P$ is its counterpart for point-objects.

The evaluation in Fig. 1 as a function of the semi-aperture angle $\theta_{\text{max}}$ was numerically performed for an excitation at $\lambda_{\text{exc}} = 760$ nm and fluorescence centered around $\lambda_0 = 580$ nm. The results show that for all practical apertures $\Gamma \approx 1$. Hence the total intensity arising from the image indeed cannot be used as a criterion for determining the phase. The intuitive explanation is that in the destructive case, the two maxima are not suppressed enough.

By the same token, the ratio between points and planes is much more favorable. $A$ and $P$ show a significant deviation from unity at practical aperture angles. For the $\theta_{\text{max}} = 64^\circ$ of a NA = 1.2

![Fig. 1. Calculated ratio between the fluorescence intensity recorded at constructive and destructive mode two-photon excitation 4Pi-confocal microscopy for a bulk $I$, ultrathin planes $A$ and point-objects $P$, as a function of the semi-aperture angle. The results for the DR-mode is denoted with a suffix. The gray background indicates the aperture angle region which is technically possible and useful for 4Pi-confocal microscopy. Available lenses are indicated by arrows. Note the desired reduction of the ratios by the DR-binary amplitude filters.](image-url)
water immersion lens, we calculated $\Lambda = 0.77$ and $P = 0.74$. For $\theta_{\text{max}} = 67.3^\circ$, present in the NA = 1.4 oil immersion lenses, the computations yielded $\Lambda = 0.73$ and $P = 0.69$. For the recently introduced NA = 1.45 oil immersion lenses [12] of $\theta_{\text{max}} = 72.8^\circ$, we obtained $\Lambda = 0.66$ and $P = 0.60$. All these values are just low enough to allow retrieval of the phase from changes in intensity.

Given the need for higher focusing angles, one might ponder the possibility to utilize spherical mirrors. However, recent thorough investigations have shown that the benefit brought about by the increase in $\theta_{\text{max}}$ is accompanied by increased spherical aberrations [10]. Therefore, we have pursued a different approach that has a similar effect, but relies on conventional lenses: the use of dark-ring (DR) binary aperture filters. We have recently shown that suitable DR-filters, placed in the entrance aperture of the two lenses, lead to a single sharp spot in two-photon excitation 4Pi-confocal microscopy [11,12]. The role of the filters is to block the illumination light in the aperture angle range of typically $(0.25\theta_{\text{max}}) < \theta < (0.82\theta_{\text{max}})$. The consequence is that the photons in the axial side-lobes are diluted in the transverse direction, so that two-photon excitation is less effective and confocal suppression enhanced.

For the same reason the disparity between the maximum intensity in the destructive and constructive imaging mode is augmented. The dashed curves in Fig. 1 reveal the theoretically predicted improvement brought about by the DR-filters, as a function of $\theta_{\text{max}}$. For the practically relevant case of NA = 1.2 water immersion we calculated $A_{\text{DR}} = 0.63$ and $P_{\text{DR}} = 0.55$. For 1.4 oil immersion we obtained $A_{\text{DR}} = 0.59$ and $P_{\text{DR}} = 0.48$. The latter result means that a point-object is only half as bright in the destructive mode. For completeness, we also give the values for the 1.45 oil immersion lens: $A_{\text{DR}} = 0.53$ and $P_{\text{DR}} = 0.37$. Hence in the DR-case, both $A_{\text{DR}}$ and $P_{\text{DR}}$ should be low enough as to allow the distinction of the phase at practical signal levels.

### 3. Experimental results

The effect of the DR-filter is demonstrated in Fig. 2 showing experimental $z$-responses of the regular and the DR 4Pi-confocal microscope in (a) and (b), respectively. The $z$-responses were measured by scanning a bright monomolecular fluorescent layer along the optic axis. We used 1.4 oil immersion lenses and the fluorescence light of 580 nm central wavelength was focused onto a detector pinhole, whose diameter corresponded to 0.65 times the Airy disk. The maximum fluctuation of the excitation power in each arm is $\sim$2.5% of the average power. For the regular 4Pi-confocal mode we measured $\Lambda = 0.76$, in good agreement with the theoretically predicted value of 0.73. In the DR-mode the value is lower: $A_{\text{DR}} = 0.63$. This also
compares well with its theoretical counterpart of 0.59.

The fact that $A$ and $A_{DR}$ is considerably less than unity can be exploited to resolve the phase difference of the illuminating beams. To demonstrate the feasibility of this method, we measured the maximum fluorescence intensities of the $z$-responses from the fluorescent layer as a function of $\phi$. In Figs. 3(a) and (b) the $z$-responses can be extracted from a horizontal line ($z$-axis). The phase was continuously varied in small increments in the vertical axis by applying a linear voltage ramp on the actuating piezo-mirror, to reveal the change of the $z$-response with $\phi$. The data are normalized to unity.

Compared with the regular 4Pi-confocal mode, the DR-4Pi, benefits from higher signal contrast, making it more robust to noise and endowing it with higher dynamic range to resolve the phase. This is depicted in Fig. 3(c), where we display the experimentally determined values of $A$ and $A_{DR}$ as a function of the phase difference. Each experimental point is based on the average of five sets of readings of the $z$-responses of Figs. 3(a) and (b). The averaging was performed in order to take into account statistical fluctuations of the laser power, a small ($<15\%$) photobleaching, and a slight axial drift of the layer. Notwithstanding these challenges, the experimental results confirm the theoretical prediction of a phase-dependent $A$ and $A_{DR}$.

4. Discussion and conclusion

The protocol for tracking down the phase is to record, for example, $n = 5$ images of the same object with $n$ identical phase steps. The image that is closest to the constructive or destructive mode is identified by the relative brightness of the point- or plane-like features in the sample. Two issues have to be considered when applying this protocol. First, potential drift of the sample and photobleaching has to be identified. Remedies for this are careful selection of the excitation intensity and restriction of the image recording to a comparatively small region. Second, one has to make sure that the intensity modulation is indeed observed from point- or planar-like structures.

Summarizing, we have theoretically and experimentally investigated the conditions under which the unknown phase difference in 4Pi-confocal microscopy can be tracked down by modulation of the intensity. An efficient implementation of this approach utilizes binary amplitude filters reshaping the effective excitation point-spread-function, such that a significant disparity arises between the brightness of thin planes and point-like objects in the constructive and destructive interference mode. Finally we note that this method should also be relevant to related interference-based methods for improving the axial resolution, such as $I^2M$ microscopy [3]; another distinct advantage is that it is readily implemented as an algorithm.
Acknowledgements

C.M.B. and S.W.H. acknowledge a postdoctoral grant by the University of the Philippines (00-74) and a project grant by the DFG (He-1977), respectively.

References