Zoological Studies 34, Supplement I (1995)

Theory and Applications of Confocal Theta Microscopy

Ernst H. K. Stelzer[1], Christoph Cremer[2] and Steffen Lindek[1, 2]
[1]*Light Microscopy Group, Cell Biophysics Programme, European Molecular Biology Laboratory (EMBL), Postfach 10 22 09, D-69012 Heidelberg, Germany*
[2]*Institut für Angewandte Physik, Ruprecht-Karls-Universität, Albert-Überle-Str. 3-5, D-69120, Heidelberg, Germany*

INTRODUCTION

The outstanding role of confocal fluorescence microscopy in modern biological research results from its optical sectioning capability, which allows three-dimensional analysis of thick specimens (Shotton 1989). This property is due to the combination of point-like light sources and detectors, such that the illumination and detection volumes are restricted. Only their common volume is relevant to the confocal observation volume. The smaller it is, the better is the resolution. The performance of a confocal microscope is therefore primarily specified by the extent of the confocal point spread function (PSF), which can be determined according to several criteria. Since confocal theta microscopy (Stelzer and Lindek 1994) provides different resolutions along the three axes, it makes sense to introduce a three-dimensional resolution criterion, e.g., the volume enclosed by the isosurface at half maximum of the PSF (VHM - volume at half maximum) (Lindek et al. 1994a). For high-resolution microscopies the VHM is in the range of al (attoliters, 1 al = 10^6 nm^3).

Even with a high numerical aperture (NA) oil-immersion objective lens, illumination and detection are performed over only about 30% of the total solid angle. This gives rise to a PSF that is elongated along the optical axis, which implies that the lateral resolution of any conventional or confocal microscope is better than its axial resolution. Therefore, especially during the past few years a lot of work has been devoted to the improvement of the axial resolution (Sheppard and Gong 1991, Sheppard and Gu 1991, Hell and Stelzer 1992, Bailey et al. 1993, Stelzer and Lindek 1994).

Recently, the development of multi-objective-lens microscopes has revealed new prospects. The 4Pi-confocal microscope (Hell and Stelzer 1992) has an increased aperture due to coherent illumination and/or detection through two opposing lenses. Therefore interference modulates the PSF along the optical axis, and resolution is improved. The confocal theta microscope (Stelzer and Lindek 1994) uses two orthogonally placed lenses to illuminate the sample and to detect the fluorescence light, thereby eliminating the elongation of the PSF along the illumination axis. Both methods have their characteristics and advantages: In 4Pi-confocal microscopy, high-NA oil-immersion objective lenses have to be used to reduce the number of interference maxima (Lindek et al. 1995). This also guarantees high spatial resolution, but will only allow the investigation of thin samples because of the use of interference and because of the short working distance. In confocal theta microscopy, the mechanical arrangement requires the use of objective lenses with a moderate NA (the maximum with water immersion is 0.94). However, water-immersion objective lenses with a long working distance can be used, so no aberrations are induced by a refractive-index mismatch (Hell et al. 1993) and one may work with large biological specimens in an aqueous medium (Stelzer et al. 1995).

A CONFOCAL THETA MICROSCOPE WITH THREE OBJECTIVE LENSES

The idea of confocal theta microscopy is to use a second objective lens to collect the fluorescence light at an angle to the illumination axis. This diminishes the overlap of the illumination and detection PSFs and thus increases the resolution (Fig. 1). The smallest extent of the PSF is reached when the detection axis is orthogonal to the i-

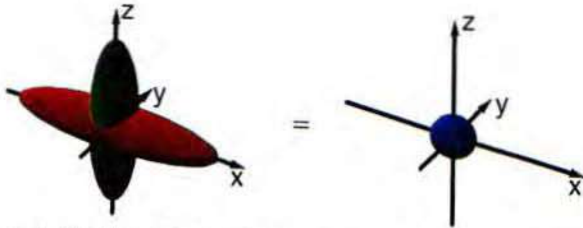

Fig. 1. The idea of confocal theta microscopy. The detection PSF (red) is orthogonal to the illumination PSF (green). Thus, the overlap is restricted to the region close to the geometrical focus, and the confocal theta PSF becomes almost spherical (blue).

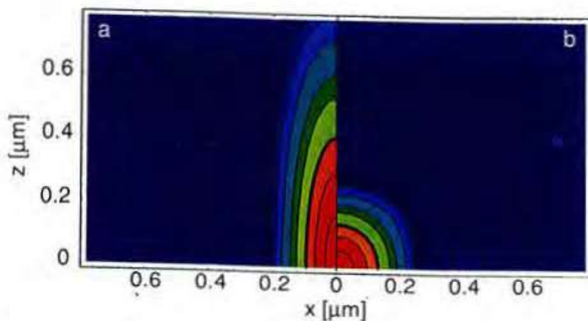

Fig. 2. Contour plots of the confocal (a) and confocal theta (b) PSFs for an orthogonal setup (illumination wavelength 488 nm, detection wavelength 520 nm, NA = 0.94).

llumination axis (Fig. 2). In practice the barrel diameter of currently available objective lenses only allows an arrangement with water-immersion lenses of NA =0.75 at an angle of 102°. (Carl Zeiss, Oberkochen, Germany).

However, this has only little influence on the resolution, and a setup with these lenses still has a four times better axial resolution and a three times smaller VHM than a confocal microscope using a lens with the same NA (for an illumination wavelength of 488 nm and a detection wavelength of 520 nm) (Lindek et al. 1994b).

An instrument with three of these objective lenses has been built at the European Molecular Biology Laboratory (EMBL) (Lindek et al. 1994b). It is a stage-scanning instrument set up on both sides of an upright bread-board (Fig. 3). The sample is suck onto a patch-clamp capillary that moves it through the common focus of the objective lenses.

The setup with three lenses is very flexible and allows several microscopies to be realized:

•Three confocal views are possible. With a single-objective-lens microscope different views are only possible if the sample is rotated (Shaw 1990, Bradl et al. 1992). However, a rotation or a deformation of the sample can cause trouble when evaluating the images. The present instrument has the advantage of allowing observation from three sides without moving the sample.

•Various theta microscopies are possible, since each objective lens can be used for illumination and for detection. The fluorescence intensity is recorded with two photomultipliers, which allows, e.g., the simultaneous imaging of two fluorophores in two different microscopy modes.

•The use of two different lasers (a pulsed tita-nium-sapphire laser that can induce two-photon absorption and an argon ion laser) increases the number of microscopies even further and allows various fluorescent dyes to be excited (Fischer et al. 1995).

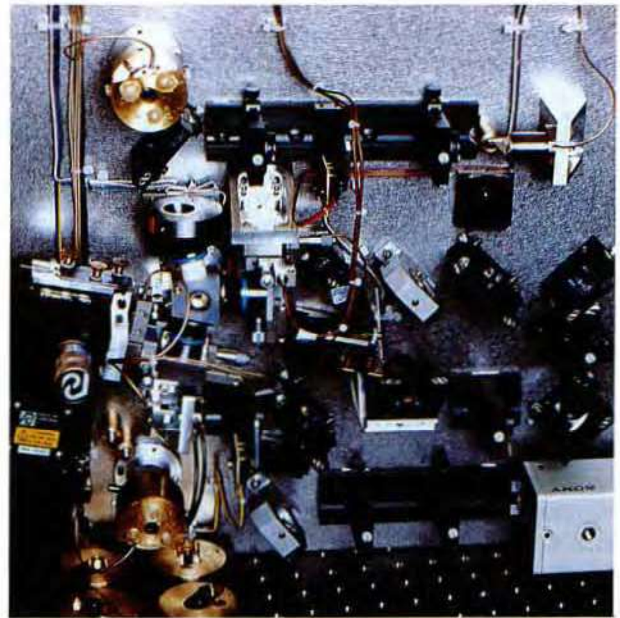

Fig. 3. Photograph of the illumination side of the confocal theta microscope.

APPLICATIONS

The microscope has been designed for biological applications with living specimens and is well suited for developmental biology:

•Since the immersion medium is water, no aberrations are introduced by refractive-index mismatch when biological samples are observed in aqueous media.

•The working distance of moderate-NA water-immersion lenses is quite large, which makes the microscope ideal for the observation of thick samples.

First measurements proved the feasibility and the resolution improvement of confocal theta microscopy (Stelzer et al. 1995). The absence of the elongation along the illumination axis and the better quality of theta images were documented with fluorescent beads. Experiments with a mouse embryo showed that biological material can be handled and that the effect of orthogonal detection also appears clearly when working with biological specimens.

CONCLUSION

Progress in three-dimensional microscopy aims at instruments with a high resolution. This is achieved with the confocal theta microscope, which provides the best resolution ever presented in light microscopy for the investigation of millimeter large biological samples.

ACKNOWLEDGMENTS

The authors would like to thank S. Albrecht, R. Pick, and G. Ritter for their help and Carl Zeiss (Oberkochen, Germany) for providing the objective lenses. S.L. gratefully acknowledges the generous support of the Boehringer Ingelheim Fonds.

REFERENCES

Bailey B, DL Farkas, DL Taylor. F Lanni. 1993. Enhancement of axial resolution in fluorescence microscopy by standing-wave excitation. Nature **366:** 44-48.

Bradl J, M Hausmann, V Ehemann, D Komitowski, C Cremer. 1992. A tilting device for three-dimensional microscopy: application to in situ imaging of interphase cell nuclei. J. Microsc. **168:** 47-57.

Fischer A, C Cremer, EHK Stelzer. 1995. Fluorescence of coumarines and xanthenes after two-photon absorption using a titanium-sapphire laser. Appl. Opt. in press.

Hell S, EHK Stelzer. 1992. Properties of a 4Pi confocal fluorescence microscope. J. Opt. Soc. Am. **A 9:** 2150-66.

Hell S, G Reiner, C Cremer, EHK Stelzer. 1993. Aberrations in confocal fluorescence microscopy induced by mismatches in refractive index. J. Microse. **169:** 391-405.

Lindek S, N Salmon, C Cremer, EHK Stelzer. 1994a. Theta microscopy allows phase regulation in 4Pi (A)-confocal two-photon fluores-cence microscopy. Optik **98:** 15-20.

Lindek S, R Pick, EHK Stelzer. 1994b. Confocal theta microscope wiht three objective lenses. Rev. Sci. Instr. **65:** 3367-3372.

Lindek S, EHK Stelzer, SW Hell. 1995. Two new high-resolution confocal fluorescence microscopies (4Pi, theta) with one-and two-photon excitation. In Handbook of Confocal Microscopy, ed. JB Pawley. New York: Plenum Press in press.

Shaw PJ. 1990. Three-dimensional optical microscopy using tilted views. J. Microsc. **158:** 165-172.

Sheppard CJR, Y Gong. 1991. Improvement in axial resolution by interference confocal microscopy. Optik **87:** 129-32.

Sheppard CJR, M Gu. 1991. Improvement of axial resolution in confocal microscopy using an annular pupil. Opt. Commun. **84:** 7-13.

Shotton DM. 1989. Confocal scanning optical microscopy and its applications for biological specimens. J. Cell Sci. **94:** 175-206.

Stelzer EHK, S Lindek. 1994. Fundamental reduction of the observation volume in far-field light microscopy by detection orthogonal to the illumination axis: confocal theta microscopy. Opt. Commun. **111:** 536-547.

Stelzer EHK, S Lindek, S Albrecht, R Pick, G Ritter, N Salmon, R Stricker. 1995. A new tool for the observation of embryos and other large specimens: confocal theta fluorescence microscopy. J. Microsc. in press.