Optik 87, No. 1 (1991) 39–40 © Wissenschaftliche Verlagsgesellschaft mbH, Stuttgart

The one-point fluorescence response in confocal microscopy

T. D. Visser, G. J. Brakenhoff

Department of Molecular Cytology, University of Amsterdam, The Netherlands

F. C. A. Groen

Department of Mathematics and Computer Science, University of Amsterdam, The Netherlands

The one-point fluorescence response in confocal microscopy. When considering the imaging process in a Confocal Scanning Laser Microscope (CSLM) in the fluorescence mode, one usually assumes that the detection sensitivity distribution is identical to the excitation distribution. We shall argue that in a fluorescence CSLM this is not the case. This leads to a somewhat narrower Point Spread Function (PSF), and thus to a better lateral resolution. Also, in agreement with this, we find that the higher spatial frequencies are less attenuated than expected from the standard approach.

Die Ein-Punkt Fluoreszenz-Antwort im konfokalen Mikroskop. Im Fluoreszenzmode der konfokalen Scanning-Laser-Mikroskopie (CSLM) nimmt man im allgemeinen an, daß am Detektor die Empfindlichkeitsverteilung identisch mit der Anregungsverteilung ist. Wir zeigen, daß dies in einem Fluoreszenz-CSLM nicht der Fall ist. Dies führt zu einer etwas schmäleren Punktbildfunktion (PSF) und damit zu einer besseren lateralen Auflösung. In Übereinstimmung dazu fanden wir, daß die höheren Raumfrequenzen weniger abgeschwächt sind als man von der Standardnäherung her annimmt.

In Fourier theory, the excitation amplitude distribution A_{ex} in the focal plane is the two-dimensional Fourier Transform of the pupil function. So for a circular lens we have for A_{ex} the well known Airy function:

$$A_{ex}(r_1) = \frac{J_1(2\pi r_1)}{\pi r_1}, \tag{1}$$

with J_1 a Bessel function of the first kind and r_1 a lateral coordinate in the focal plane (fig. 1).

Because the confocal lens turns the incoming homogeneous plane wave with amplitude A_0 into a converging spherical wave, the energy flux is smeared out. Assuming that the confocal lens obeys the sine condition, implicates that we are dealing with the so-called aplanatic energy projection over the spherical wave front [1]. This means that an annulus with area δS_0 of the incoming plane wave is projected onto a ring shaped part of the spherical wave with area δS_1. One can easily show that [1]

$$\delta S_0 = \delta S_1 \cos\theta, \qquad (0 \leqq \theta \leqq \Omega) \tag{2}$$

Received October 11, 1990.

Taco D. Visser, G. J. Brakenhoff, Department of Molecular Cytology, University of Amsterdam, Plantage Muidergracht 14, 1018TV Amsterdam, The Netherlands.

F. C. A. Groen, Department of Mathematics and Computer Science, University of Amsterdam, Kruislaan 409, 1098SJ Amsterdam, The Netherlands.

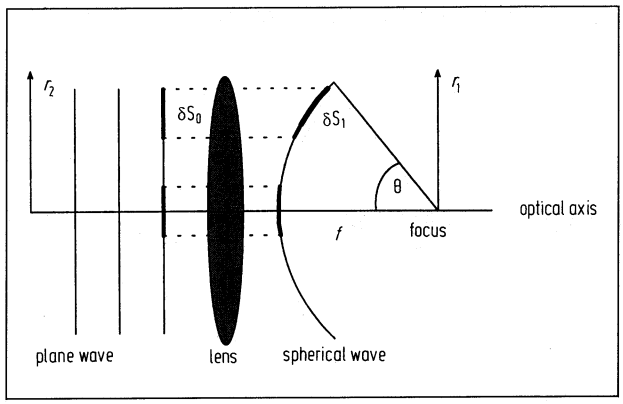

Fig. 1. Model of an epi-fluorescence CSLM. Indicated is the aplanatic energy projection. r_1 and r_2 are dimensionless lateral coordinates.

where Ω denotes the semi-aperture. Conservation of energy then yields for the amplitude on the spherical wave front

$$A_0^2\,\delta S_0 = A^2(\theta)\,\delta S_1\,. \tag{3}$$

So the excitation light travelling to focus under an angle θ with the optical axis, will have the following angular dependence of the amplitude

$$A(\theta) = A_0\sqrt{\cos\theta}\,. \tag{4}$$

The point of this paper now is that the fluorescence light is emitted as an isotropical spherical wave with amplitude A_{flu}, and has no $\cos\theta$ dependence as in eq. (4). During the imaging of the fluorescence light the reverse of the above geometrical process takes place at the confocal lens. Now the energy flux through an area δS_1 of the spherical fluorescence wave is concentrated onto an area δS_0 of the emerging plane wave. So the plane wave behind the lens is not uniform, but its amplitude A_{col} varies with θ as

$$A_{col}(\theta) = \frac{A_{flu}}{\sqrt{\cos\theta}\,\cdot} \tag{5}$$

The sine-condition reads

$$r_2 = f\sin\theta\,. \tag{6}$$

It means that the emergent rays leave the lens at the same distance from the optical axis – which we called r_2 – as the corresponding ray entered it in the object space. With the help of eqs. (5) and (6) the pupil function of the collector

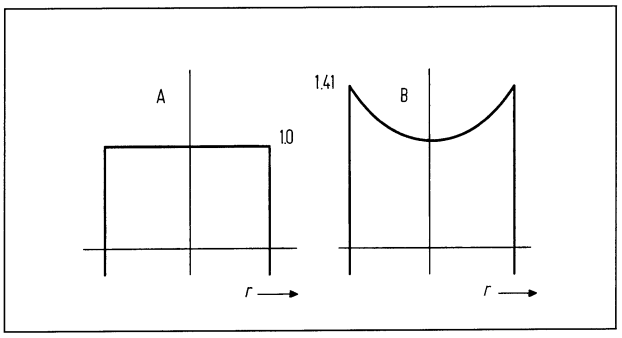

Fig. 2. The pupil function for the illumination (A) and that for the detection (B), for a semi aperture angle $\Omega = 60°$.

lens for fluorescence radiation, when expressed in r_2, effectively has the form

$$P(r_2) = 1/\sqrt{\cos(\sin^{-1}(r_2/f))} \quad (0 \leqq r_2 \leqq f\sin\Omega)$$
$$P(r_2) = 0 \quad (r_2 > f\sin\Omega) \tag{7}$$

with f being the focal length of the lens (see fig. 2).

The detector amplitude sensitivity distribution $K(r_2)$ is the Fourier-Bessel Transform of this function

$$K(r_2) = 2\pi \int_0^{f\sin\Omega} \frac{\varrho}{\sqrt{\cos(\sin^{-1}(\varrho/f))}} J_0(2\pi\varrho r_2)\,d\varrho\,, \tag{8}$$

with J_0 a zeroth order Bessel function of the first kind. The intensity detection distribution is the square of $K(r_2)$, this function is plotted in fig. 3 for a semi-aperture of 70°. Notice that the full width at half maximum (fwhm) is now about 4% less than that of the Airy disc. The sidelobes however are somewhat higher.

We will assume that the emitted fluorescence is proportional to the excitation intensity. The overall PSF can then be written as the product of the excitation intensity distribution and $K(r_1)^2$:

$$PSF(r_1) = \left(\frac{J_1(2\pi r_1)}{r_1}\right)^2 K(r_1)^2\,. \tag{9}$$

The conventional approach to fluorescence confocal microscopy is due to Wilson and Sheppard [2]. Here one assumes the same pupil function for excitation and detection [1], in other words $K(r_1)^2$ is taken equal to the Airy intensity distribution. Comparing this with our method we see that for a semi-aperture angle of 70° the fwhm of the PSF is now about 2.5% less. However, the first sidelobe is 30% higher, although it still reaches to a mere 0.4% of the central peak.

Let us now define the Optical Transfer Function of our system as the Fourier-Bessel transform of the PSF. The results are shown in fig. 4 for $\Omega = 70°$. We find the same cut-off frequency as in the conventional approach, but the transmittance at half the cut-off frequency is now 8% better. Also we find a significant increase (up to 25%) in the intensity transmission at the higher spatial frequencies, as shown in the inset of fig. 4.

[1] If one takes the difference between the two wavelengths into account, there would still be a difference in the form of the two pupil functions.

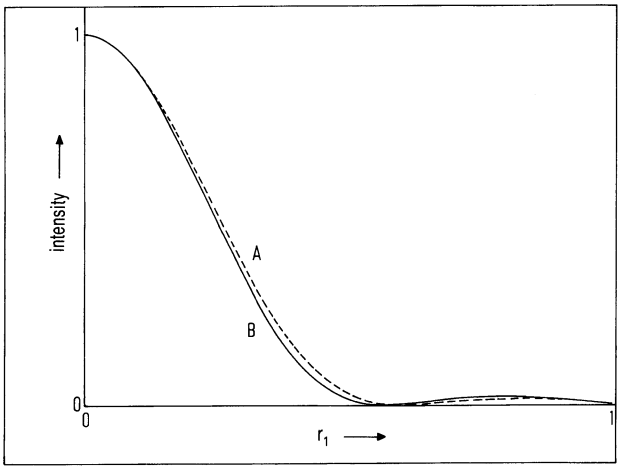

Fig. 3. The Airy intensity distribution (A) and the intensity detection distribution in the focal plane (B). The latter is depicted for a semi-aperture angle of 70°.

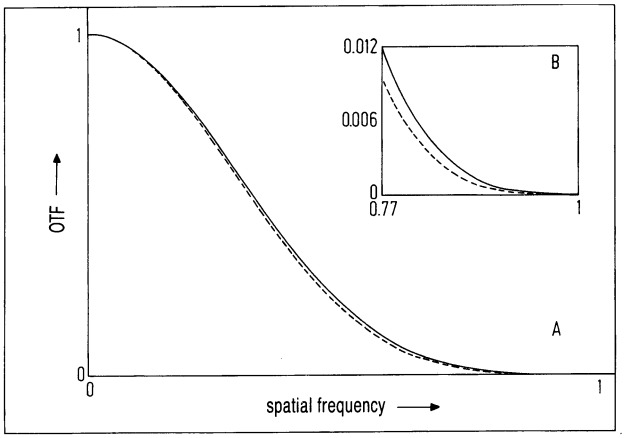

Fig. 4. A: The Optical Transfer Function for a fluorescence CSLM, with a semi aperture angle $\Omega = 70°$, our approach (solid line) and the conventional approach (dotted line). B: Detail of the same transfer function, showing a better transmittance of the high spatial frequencies in our approach (solid line) than in the conventional one (dotted line). Both OTF (0) and the cut-off frequency are normalized to 1.

In conclusion we may say that the standard Fourier approach underestimates the lateral high frequency transmission characteristics of fluorescence confocal microscopy. In addition one may expect that the increased fluorescence amplitude at the pupil's edge will slightly reduce the axial resolution. This in accordance with the well known axial resolution reduction when annular apertures are used [3].

References

[1] J. J. Stammes: *Waves in focal regions.* Adam Hilger, Bristol 1986.
[2] T. Wilson and C. J. R. Sheppard: *Theory and Practice of Scanning Optical Microscopy.* pp. 153–155. Academic Press, London 1984.
[3] G. J. Brakenhoff, P. Blom and P. Barends: *Confocal scanning light microscopy with high aperture immersion lenses.* J. Microscopy **117**, Pt. 2 (1979) 219–232.