Application Letter 11

Significance of the Pinhole in Confocal Microscopy

A Discussion of Pros and Cons

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Table of Contents

Note:

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1. Function of the illumination pinhole

In confocal microscopy the illumination pinhole represents a point light source that is projected, subject to the diffraction effect, through the microscope objective onto the focal plane.
Leica confocal microscope systems use the illumination pinhole in combination with a glass fibre. Thus a very stable coupling is attained between excitation light and system and a point light source can be defined via the pinhole.
The glass fibre coupling, first introduced by Leica, has proved reliable to such a degree that meantime almost all producers of confocal systems are taking advantage of this beam coupling method.
The illumination pinhole itself serves as an absolute reference point to all subsequent optical components. If a true illumination pinhole is used, lateral shifting of the laser light, as it may be caused by thermal or mechanical influences, only leads to a decrease in light intensity. If there is no illumination pinhole installed, lateral shifting of the laser light may cause alterations of the entire beam path which then has to be completely readjusted.
The Ar- and Ar/Kr-lasers which are typical of confocal microscopy often do not supply the required beam quality. Owing to the diffraction effect an illumination pinhole ensures that the disadvantageous intensity profile produced by these lasers is replaced with a favourable intensity profile.
The diffracted image generated by the illumination pinhole shows no axial aberration of the single point light sources and in addition is also very useful for a precise adjustment of the system. That means, that virtually one point (the main maximum) is projected from the pinhole plane to the sample.
The illumination pinhole (point light source), the light spot on the object plane as well as the detection pinhole (point light detector) exactly lie on the optically conjugated planes and therefore are precisely aligned to each other. This configuration exactly corresponds to the “confocal” arrangement.

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Info box:

Chromatic aberrations, as they occur for physical reasons when using optics simulating an excitation pinhole, are completely avoided by the illumination pinhole. Axial chromatic aberrations in the simulated illumination pinhole are projected in reduced size into the focal plane according to the following equation:

$$CAF = \frac{CAD}{(M_{obj} \bullet M_{confocal})}$$

with CAF= maximum distance of focal positions in the focal plane,
CAD = maximum distance of focal positions of the simulated illumination pinhole,
M_{obj} = magnification of the objective used
$M_{confocal}$ = magnification of the confocal optics used

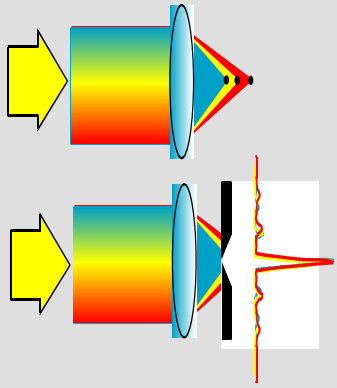

Figure 1: Avoiding varying axial positions of the point light source in case of different wavelengths of the excitation light

Stray effects, which possibly occur prior to the excitation light coupling, are effectively suppressed by the illumination pinhole functioning as a spatial filter.

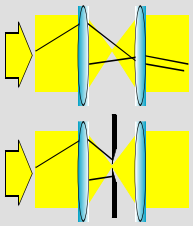

Figure 2: Effect of the illumination pinhole as an ambient light filter (suppression of stray light)

2. Function of the detection pinhole

The detection pinhole in confocal microscopy functions as a point light detector and, depending on the aperture size, suppresses light coming from outside the focal plane.

The suppression of lateral stray light and of blurred image parts from different planes enables contrasty high resolution imaging of the sample to be examined. The larger the diameter of the detection pinhole, the more light is detected from areas outside the depth of focus.

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The smaller the diameter of the detection pinhole, the thinner is the detected optical section and the more defined and contrasty are the microscopic images. Unfortunately, reducing the pinhole diameter also diminishes the intensity of the detection signal. Thus there is a limit where reducing the pinhole diameter stops making sense. Consequently, a compromise has to be found between the conflicting aims of ***minimum*** thickness of the optical section and ***maximum*** detection signal. The diagram depicted below illustrates this conflict.

3. Resolution effects

The theoretical resolution limit as it is defined by Abbe's equation cannot be achieved by a real microscope for two reasons. First, all optical components have inherent flaws, which may yet be minute. Secondly, the virtual resolution can be deteriorated by various noise phenomena which results in worse *visualized resolution*. The diagram below illustrates this effect.

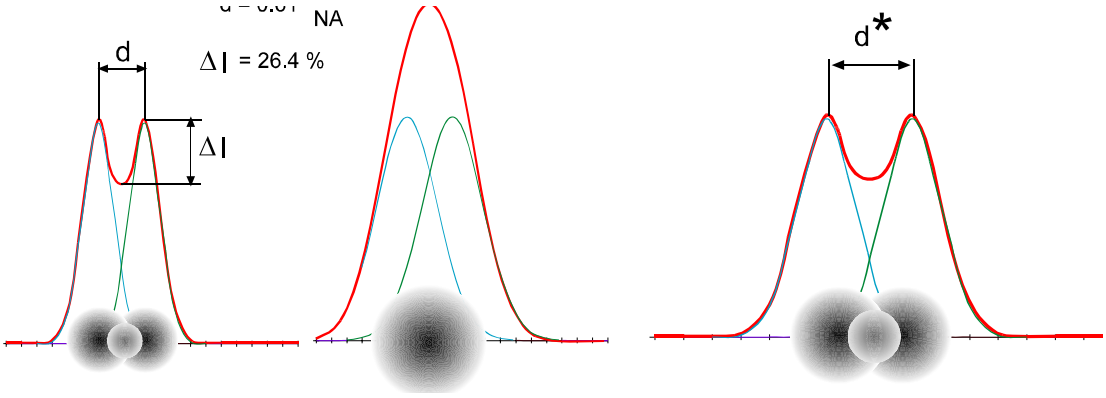

Figure 4: Resolution criterion according to Rayleigh
Left: structure resolved (without broadening by noise)
Middle: structure not resolved (broadening by noise)
Right: transmitted, virtually visible resolution of the structure diminishes

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4. Historical reasons for the use of several detection pinholes

Originally confocal microscopes were equipped with two detection pinholes in order to compensate for the imperfections of photomultiplier detectors in the red spectral range. Compared to modern photomultipliers, the formerly available photomultipliers showed about 3 times poorer response properties in the wavelength range from 600 nm upward.
With the object of increasing the detection probability within the red spectral range, a separate pinhole was installed in front of the detector. With the pinhole set to larger aperture sizes, more light could enter the detector through this pinhole, so as to improve the signal-to-noise ratio. This, however, caused an increase of blurred image parts within this detection range.

5. One single pinhole or several pinholes ?

Due to the differing spherical extension of the point-spread-function, the focus volume is not constant when detection wavelengths vary.
Using several detection pinholes with different aperture diameters is an approach to keep constant at least the thickness of an optical section for all wavelengths.
With closed detection pinhole the thickness of the optical section depends linearly on the emission wavelength

$$d_{opt.\sec t.} \propto \lambda_{emission}$$

With a typical application such as the simultaneous detection of FITC and TRITC the emission wavelengths of the two fluorochromes lie at 530 nm and 580 nm. The difference in the emission wavelengths enters linearly into the thickness of the optical sections and results in a maximum difference of 8% of the optical sections. Normally, however, the detection pinhole will not be closed but set to the diameter of an Airy disk. Both the diameter of the detection pinhole and the emission wavelength are taken into account for calculating the thickness of the optical section. The effects of the unclosed pinhole on the thickness of the optical section reduce the wavelength-dependent influences. Standard settings normally cause differences of only 3% in the thickness of optical sections.
In order to attain a constant thickness of the optical section with longer detection wavelengths, the pinhole diameter has to be reduced. Thus only photons of the defined section enter the pinhole. It is interesting that this is exactly the opposite procedure to that applied when several detection pinholes were introduced (see above description).
Yet, the signal-to-noise ratio decreases with longer detection wavelengths, which necessarily deteriorates the visualized lateral resolution, as well. The effects of a low signal-to-noise ratio on the visualized lateral resolution are shown in figure 1 (see page 5).

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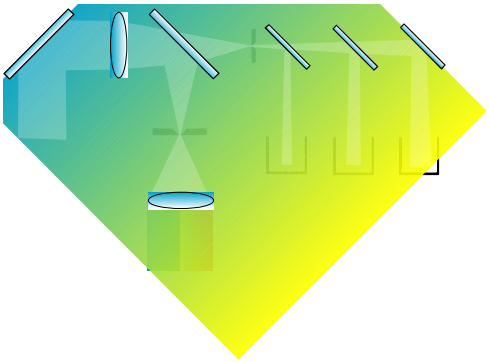
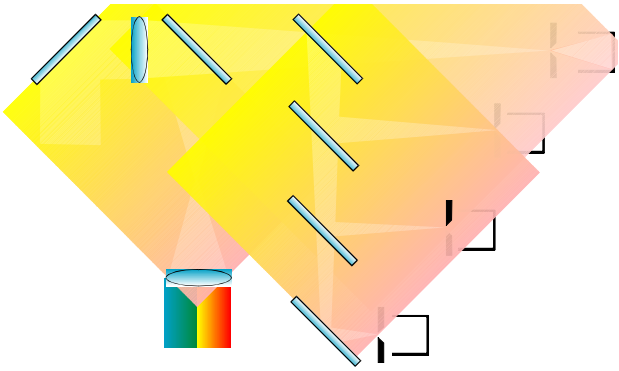
One of the problems that occur when several detection pinholes are used, is the question of precision and long-term stability of the system. Part of this problem is the expected lateral aberration (pixel aberration) of the single channels which can only be balanced by time-consuming readjustment and/ or software corrections. Much more noticeable are the intensity and resolution losses which accrue from a laterally maladjusted detection pinhole.This problem is completely avoided, if a single detection pinhole is used.

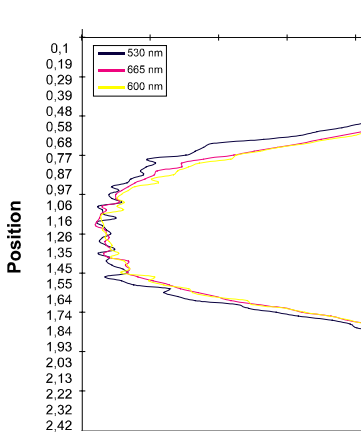


Scheme of a single pinhole detection system



Scheme of a multipinhole detection system

A single pinhole detection system meets the basic requirements to ensure continuous and reproducible precision, as it is absolutely necessary, for example, to carry out ratio measurements in physiology or colocalization analyses in cell biology.
An evidence of the precision of the single detection pinhole can be shown by the following application.



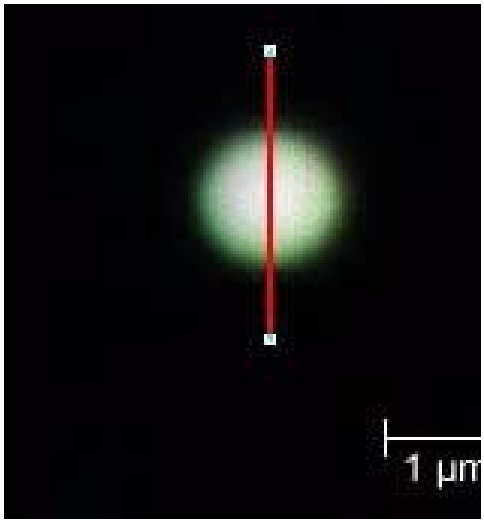


Figure 5: Beads in xy-section
Optimum superimposition of all detection channels (red, green, blue) to one white overall image.
Thus the collocation of a point-shaped structure can be tested in a confocal system

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The above application image shows beads of 1 µm, which were stained with fluorescence colours.

Optical cross sections of these beads are recorded by means of the dominant Ar/Kr-lines of 488nm, 568nm and 647nm. As lateral aberrations cannot occur due to the simultaneous detection and the use of one single detection pinhole, software corrections are no longer necessary. The Leica TCS NT system implements sequential image recording definitely free from lateral aberrations, provided optical components between the two pinholes are not changed between the single shots.

Lateral and axial aberration in confocal operation can easily and clearly be discerned with the beads. Lateral aberrations are indicated by a unilateral colour edge at the beads. A colour border positioned concentrically around the bead discloses different axial and lateral resolutions of the single wavelengths. If there is no colour border, it can be concluded that resolution differences are not detectable any more.

The correction of lateral aberrations having occurred in a multipinhole detection system implies time-consuming readjustments and intensive computer calculations. Readjusting a maladjusted multipinhole installation is a delicate task. Manual or automatic adjustment is dependent on intensity alterations which occur when pinhole positions are changed. The diagram below is to show how different positions can accrue from detecting the sample without any intensity alterations.

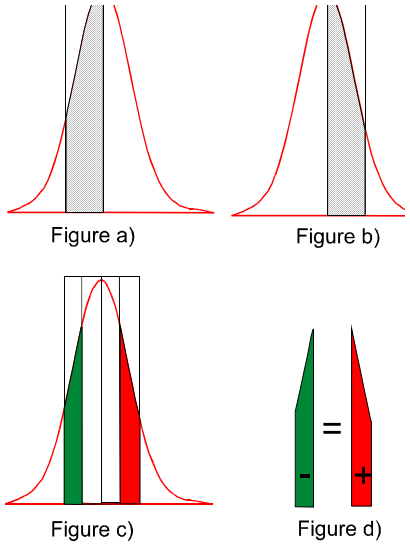

Figure 6: Poorly centred detection pinhole; the hatched area corresponds to the intensity detected.
figure a): sample is detected at position A
figure b): sample is detected at position B
figures c and d): Intensity differences relative to the centred detection pinhole are identical. Intensity differences between both decentred positions do not exist

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Very small pinhole apertures are necessary to improve the localization precision for adjusting detection pinholes. As a result the detectable intensity decreases correspondingly. Adjustment signals, if to be suitable at all for adjustment, must have certain minimum intensities, which can hardly be expected with fluorescent samples. For that reason a special sample has to be used for the adjustment, even if fully automatically executed.
Apart from the fact that the pure intensities after the detection pinhole indicate nothing about the real detected positions, adjustment of a multipinhole detection system means enormous work.

In Leica TCS-NT systems the spectral splitting of the detection light takes place **after** the detection pinhole. This solution does not exact high precision for positioning and moving the beam splitters. Slightly tilting the beam splitter out of the original position is uncritical, due to the large aperture diameter of the detector.

With multipinhole detection systems the beam splitting is executed **between** the virtual illumination pinhole and the detection pinhole - i.e. exactly within the sensitive optical area where confocality should be guaranteed.
A four-channel system of a multipinhole detection system has at least four beam splitters installed between the virtual illumination pinhole and the last detection pinhole. Slightly changing the position of one of these beam splitters has direct effects on the subsequent optical components. Here a slight drift cannot be compensated for by a large detector aperture, since the detection light has first to be directed through the individual detection pinholes.
Multipinhole detection systems therefore react very sensitively to beam splitter positions (manufacturing tolerances, aging processes, adjustment inaccuracies). Manufacturing tolerances as well as long-term drifts of the beam splitter position, as they occur, e.g. due to warming up, make it necessary to readjust the system after a filter exchange, in order to lead the detection light through the pinholes. Consequently a quick filter exchange is practically impossible.
The question arises then what the real improvements are of using several detection pinholes?

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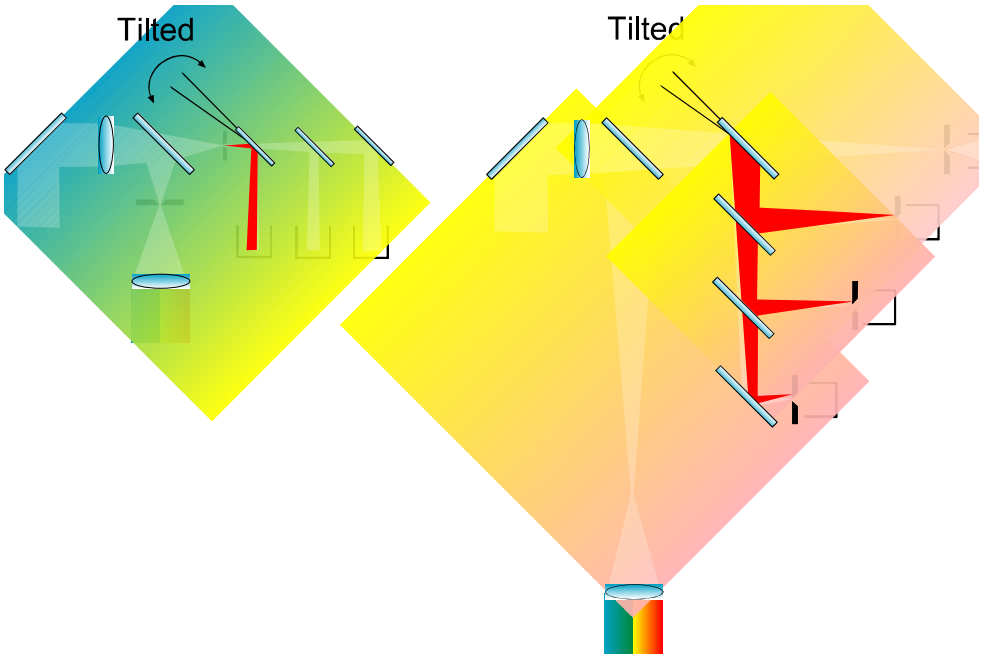

Figure 7: Effects resulting from tilting a beam splitter on a single pinhole detection system and on a multipinhole detection system

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6. Summary

With illumination pinhole	Without illumination pinhole
Precise point light source for all wavelengths	Depending on the quality of the optics more or less focus aberrations of the different wavelengths (several point light sources -> several illumination foci in the sample)
Reference point for the optical installation, main maximum of the generated diffraction-limited image can be used for easy adjustment	Position of the point light sources are difficult to define for adjustment purposes
Illumination pinhole serves as spatial filter and improves the intensity profile of the beam	Unfavourable laser modes are directly projected into the confocal optical part of the system
One single detection pinhole	**Multipinhole detection**
Lateral aberration ruled out	Lateral aberration can only be compensated for by time-consuming adjustment and intensive computer calculations
Simple adjustment	Intricate adjustment Systems with multipinhole detection have to adjusted as a routine measure (about once a day), i.e. there are intervals when the system is maladjusted. Thus the colocalization of different signals can no longer be guaranteed
Beam splitter after detection pinhole	**Beam splitter in front of detection pinhole**
In case of slight inaccuracy of the beam splitter position, stability is guaranteed due to large detector apertures	Very sensitive to alterations of the beam splitter position, readjustment of the pinholes necessary
Quick filter exchange possible during an experiment, without need for readjustment	To ensure identical detection positions after filter exchange, pinholes have to be recalibrated by means of a reference sample

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