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Why do we need a Pinhole in a Confocal Microscope?

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Whenever we use a confocal microscope and when we will have to discuss its parameters and features, inevitably the pinhole will be talked about and how its size effects the resulting images. This brief introductory text explains the meaning of the pinhole and is meant for readers who do not want to spend a lot of time studying the detailed theory of confocal microscopy, but still want to have an idea of how the instrument works and which limitations it has.

What is a Pinhole?

Optical lenses are essentially characterized by two parameters: the curvature of the lens' surface and the diameter. The curvature determines in which directions the rays are refracted and the diameter defines how many rays will contribute to the resulting image. In the simplest case, the beam diameter is given by the lens' edge. However, in many cases though, the beam diameter is controlled by a separate diaphragm. Iris-diaphragms, which are composed of a number of lamellae, are commonly used. The diameter is changed by mechanical adjustment of that lamella, resulting in a stepless control of the resulting diameter. The term iris-diaphragm refers to the function of the iris in the human eye. It is named after Iris, the ancient Greek goddess of the rainbow.

Very small diaphragms (apertures) are tricky or impossible to make with this lamella-technique. The simplest method for generating a very tiny aperture is quite trivial: take a piece of cardboard or aluminum foil and prick a tiny hole with a sewing needle. That hole can be called then a "pinhole". With such a pinhole you can make a camera obscura yourself – without any lenses [1].

What is the effect of a pinhole in a confocal microscope?

Confocal microscopes are supposed to separate optical slivers out of the specimen and record that as an image. In the best case, these small slices are about the thickness of the microscope's depth of focus. To that end, the sample is scanned by a light spot which is made as tiny as possible – comparable with an electron beam scanning across the screen of a vintage television set. The tiniest spot we can generate by ordinary optical means has a "diffraction limited" diameter: the diameter is defined only by the color of the light (wavelength) and the opening angle (numerical aperture) of the lens. It is the image of an infinitely small light point. The intensity-distribution in the focal plane is consequently called the "point-spread-function" (psf). As a matter of fact, the psf is a three-dimensional structure and it defines the resolution in all three spatial directions. A representation of this intensity distribution in the focal plane is shown in Figure 1. In the center, we can see a bright spot, the Airy-disc. Further out we can recognize concentric rings. As conventional light sources are not infinitely small points, but have a finite dimension, their light is focused onto a tiny aperture – just a pinhole, which then acts as a good approximation of a point-shaped light source. Usually, we do not discuss the pinhole concerning the illumination part: laser light has ideal properties to be focused to a diffraction limited spot – entirely without the use of a pinhole.

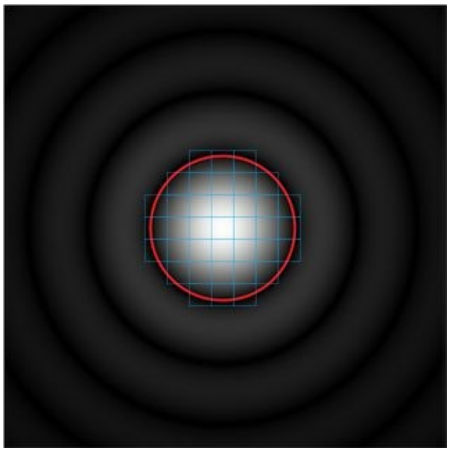

Fig 1. Airy pattern and pinhole size

To generate an optical slice, the sensor must scan the sample synchronously with the illumination. The detection function, as well, must resemble a diffraction limited spot which is at all the times overlaid with the illumination spot during the scanning process. We can generate such a diffraction limited perception-function, if we thread the emitted light through a tiny aperture: the detection pinhole. This pinhole is the one commonly referred to when the pinhole and its diameter in a confocal microscope is discussed.

The Term "confocal" refers to this arrangement of the illumination and detection spot. Both are focused onto the identical point [2], thus the focal spots are overlaid. The image is then generated by scanning the whole field of view spot by spot. The scan procedure usually is performed with a set of scanning mirrors. Such "scanners" are commonly known from the cash register at the supermarket and from laser shows in the disco.

In Figure 2 [3], we can see how the detection pinhole cuts away all of the signal not emanating from the focal plane. Therefore, it is also called a "spatial filter" which filters all sharply reproduced fractions and suppresses the extrafocal signal. Because such an instrument cuts optical slices out of a prepared sample, it is also called "optical knife".

Why is the pinhole diameter variable?

Similar to the illumination, the intensity distribution in the intermediate image plane is an Airy-Pattern (Figure1). The pinhole will – depending on its diameter – transfer variable fractions of the pattern to the detector. Usually, for standard confocal imaging, it is recommended to fit the size of the pinhole just to transmit the Airy-disc. This diameter is named the "Airy-Unit" (AU). The diameter of that disc in the intermediate image plane depends on the color of the light, numerical aperture of the objective lens, magnification of the objective lens, and the magnification of internal relay optics. The NA and the lens' magnification are engraved on the lens' barrel and will change, when the objective is changed. Consequently, the recommended pinhole diameter is different, when a lens with different NA and/or magnification is used. Similarly, the diameter should be changed, when blue, green or red emission is recorded.

Furthermore, a variable pinhole provides freedom to increase optical section sharpness by reducing the diameter. Or a thicker section can be recorded by increasing the diameter.

What happens if the pinhole is enlarged?

As indicated above, the pinhole diameter for common confocal imaging should just transmit the Airy-disc pattern. That result is a good and practical compromise, not a law of nature. If the diameter is increased, then more and more contributions from outside the focal plane will reach the sensor and gradually fog the sharp image [4]. The apparent brightness of the image will, therefore, increase and one could get the impression that the signal-to-noise ratio has improved. But the increase in brightness is only due to the addition of unsharp, unwanted fractions. As the pinhole diameter approaches infinity (is removed from the beam path), then the microscope behaves like an ordinary non-confocal, widefield microscope (see Figure 4). Therefore, it is generally not recommended to use a pinhole above 1 AU, just to reduce the statistical noise in the image.

The lateral resolution is not affected for a pinhole diameter increasing above 1 AU. It will always stay at a value close to the resolution of a normal widefield microscope and correspond to Abbe's resolution limit d=λ/2NA (Figure 5).

… and if it is decreased?

The thinnest optical sections are calculated at the diffraction limit for the axial direction when the pinhole diameter is assumed to be zero. Of course, that assumption is not practical, for no signal at all will then pass the pinhole. The theoretical slice thickness is about 25% thinner [4] for a pinhole with zero diameter as compared to one Airy-Unit.

Contrary to pinhole diameters above 1 AU, the lateral resolution is affected by smaller pinhole sizes as well. At pinhole of zero, we can expect an improvement in resolution [4] of about 30%. We can take advantage of this effect, at least partially, by closing the pinhole somewhat below 1 AU. As an example, if we use a pinhole diameter of 0.6 AU, we can already attain a 60% potential gain in resolution. For securing a good signal-to-noise ratio at smaller diameters, we can use systems with high efficiency for collecting the emitted fluorescence signal and nearly noise-free detectors, like the TCS SP8 confocal laser scanning microscope with hybrid detectors (HyDs) offered by Leica Microsystems.

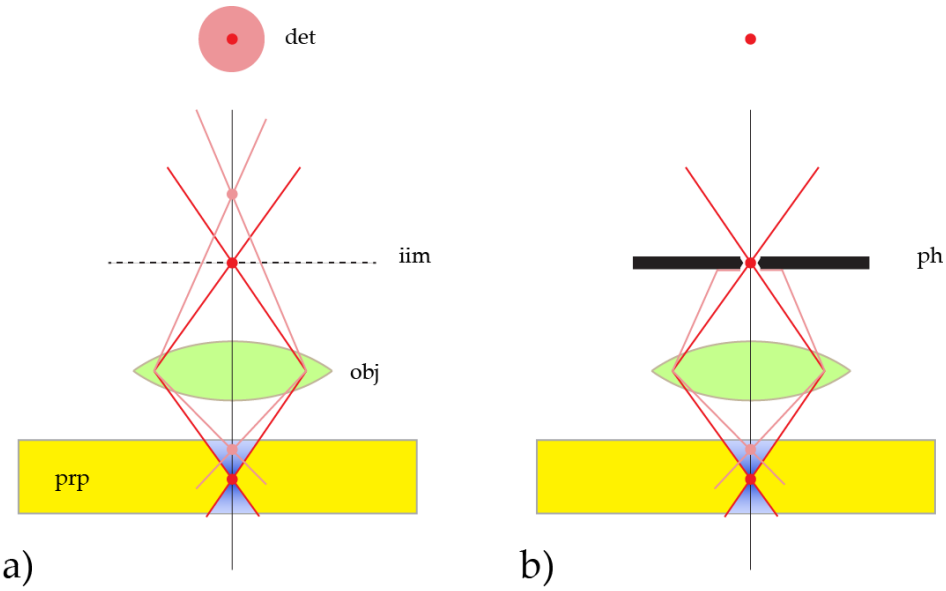

Fig 2: The optical knife.
a) in a compound microscope, an intermediate image (iim) is generated by an objective lens (obj). The intermediate image contains signals both from the focal plane (red) and extrafocal space (pink) that have been emitted from the sample (prp). If a single point is observed, then the detector (det) will perceive both a point-shaped object from the focal plane and extended unsharp discs from other regions.
b) upon insertion of a pinhole (ph) in the intermediate image, nearly all the signal from the extrafocal space is cut off and only emission from the sharp focus will reach the detector: the spatial filter generates an optical section.

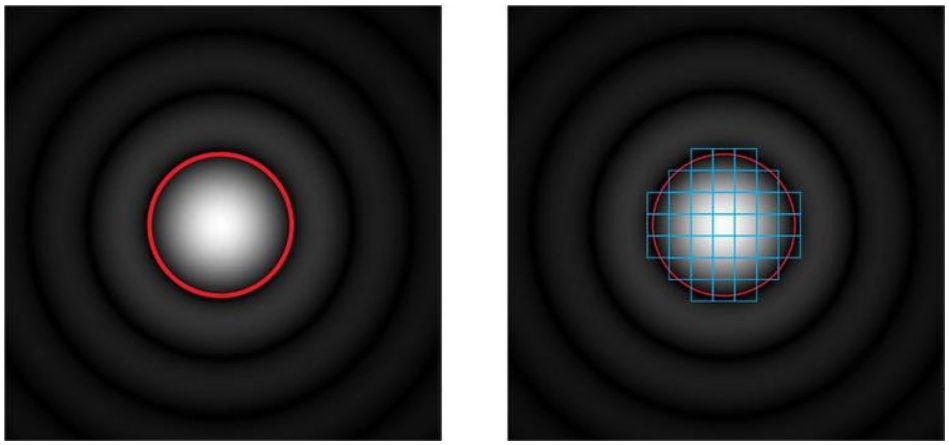

Fig 3: Pinhole diameter and diffraction pattern. Left: the red circle corresponds to a circular pinhole set to 1 AU. Such a pinhole will just cut the inner disc of the diffraction pattern and generate an optical section of a thickness (dz). Right: With image-rescanning (see text further below) a series of subsets of the diffraction pattern (blue boxes) is recorded separately. Afterwards, this information is calculated by computer algorithms into a new image, providing a better lateral resolution than wide-field microscopy. The thickness of the optical section always corresponds to a pinhole area which equals the total area of all boxes rescanned. In this example, the area is similar to the pinhole area and the thickness will be the same as for the pinhole.

How does that compare to psf-subdivisions with image rescanning methods?

An alternative to increasing the lateral resolution by shrinking the pinhole is a method called "image rescanning" [5], as well as a number of differently named similar approaches. Here, analogously, the diffraction pattern is scanned a second time and the signals are then distributed to the appropriate pixels (Figure 3, right). By computing a new "synthetic" image, the lateral resolution is improved by about a factor of 1.4, in comparison to Abbe's limit for widefield microscopy, roughly corresponding to the resolution of a confocal microscope with a pinhole diameter of zero. For this method, the lateral resolution is more or less independent of the rescanned diffraction pattern area. Therefore, the images might become

somewhat brighter. It is important to keep in mind, though, that for one AU already more than 80% of the maximum brightness is achieved (light rays collected).

However, the performance for optical sectioning decreases with larger areas of the rescanned diffraction pattern. It behaves like a comparable larger area of a pinhole (see blue vertical lines in Figures 4 and 5). By applying a larger area of the diffraction pattern, the desired advantage of a scanning microscope would be nullified, i.e., the generation of thin optical sections. These limitations would put the considerable investment for such a raster microscope into question.

Literature:

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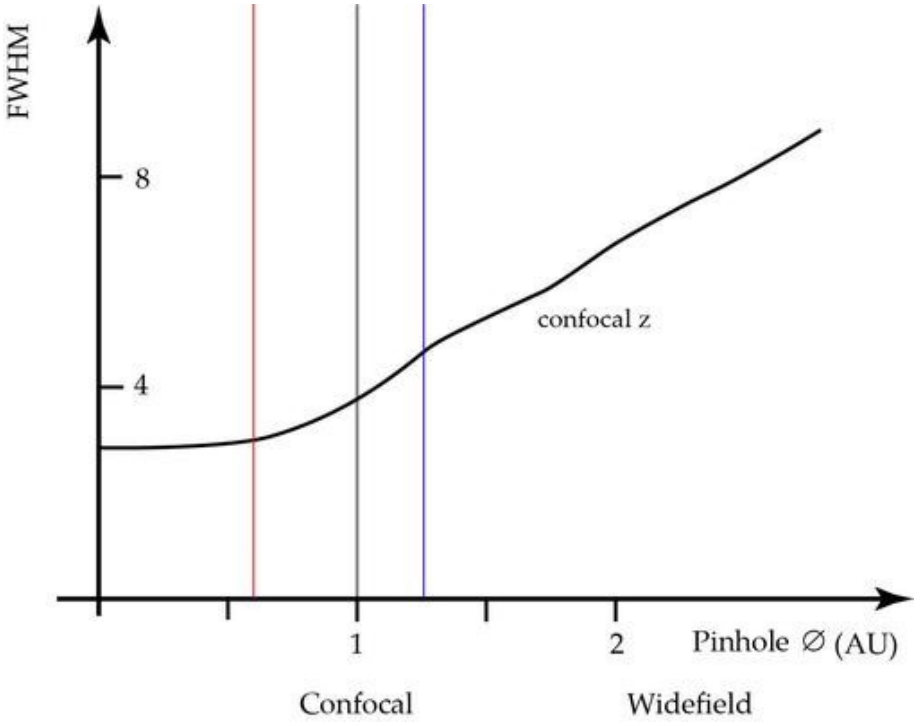

Fig 4: Thickness of optical sections as a function of the pinhole diameter. A good and practical setting for most cases is a diameter of 1 AU (gray vertical). For increased diameters, the detector will sense additional signal that comes from outside of the focal plane. As a result, the performance of optical sectioning is deteriorated, although the image appears brighter. For diameters below 1 AU, the section becomes thinner, but it will not pass below a finite, diffraction limited value (for the diameter approaching 0). As an optical slice has no sharp borders, like a slice of bread, the thickness is given in multiples of the full width at half maximum (fwhm).

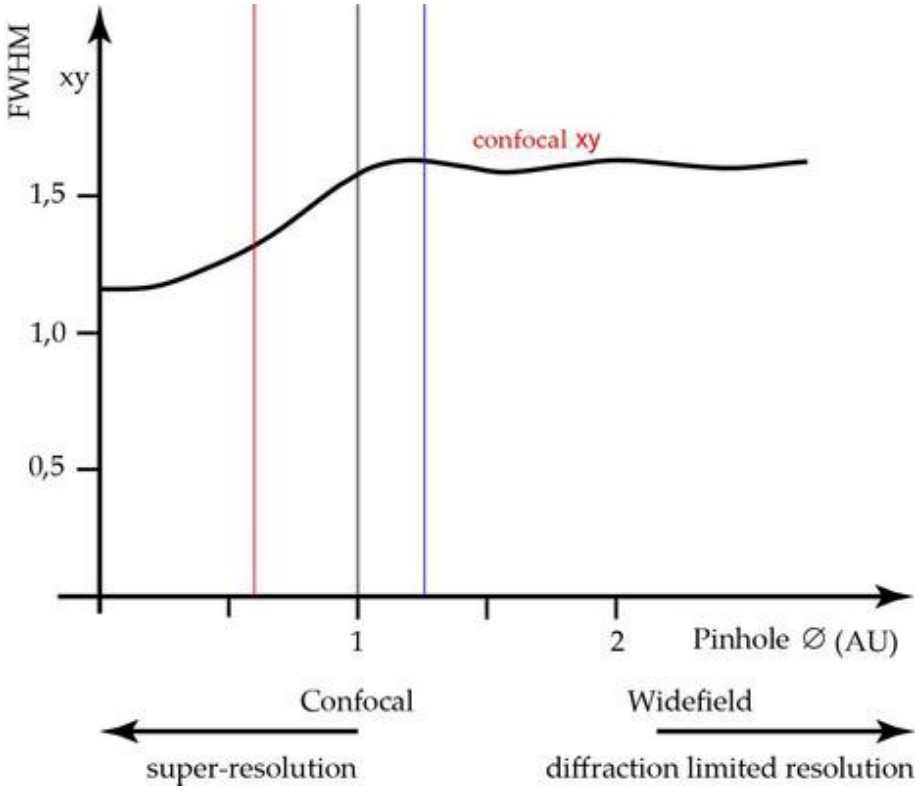

Fig 5: Lateral resolution as a function of the pinhole diameter. For diameters of 1 AU (gray vertical line) and above, the lateral resolution is more or less constant and equals that of conventional wide field microscopy (diffraction limited resolution). For pinhole diameters below one AU, the lateral resolution improves. Maximal improvement occurs at a pinhole diameter of zero and then amounts to some 30%. Already at diameters of 0.6 AU, about 60% of the potential improvement is exploited (red vertical line).