Scanning Optical Microscopy

3.1 "Classical" Optical Microscopy

- Basic definitions

The term used in a title is rather uncertain. In microscopy the recent progress of optical methods resulted in the appearance of a large number of techniques many of which can be considered as classical. However, the purpose of this chapter is not to review the wide capabilities of optical microscopy but define some terms which will be used further for the explanation of a confocal microscope principle of operation. We introduce these basic concepts taking the widefield optical microscope as an example **(Fig. 1)**.

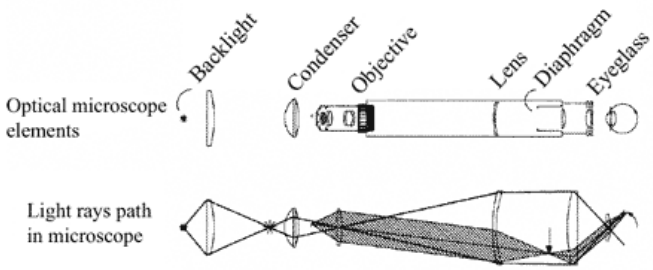

Fig. 1. "Classical" optical microscope schematic diagram

In such a microscope, the image of an object placed in a uniformly illuminated field of view is projected by an optical system onto the retina of the eye or onto a sensor (e.g. CCD array in a video camera) plane. Generally, a sensor receives the light emitted by different areas of a specimen which are both in a focal point of an objective lens and beyond a focal point **(Fig. 2)**.

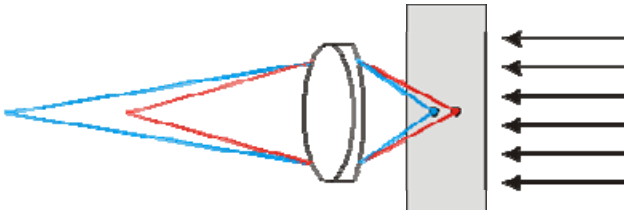

Fig. 2. In a widefield microscope, various points of an object are viewed simultaneously, therefore points of planes, other than the object plane, produce background illumination lowering the contrast

- Numeric aperture and dimensionless units

To describe parameters of optical instruments, some special terms are adopted in optics. Namely, numeric aperture NA is defined as

$$NA = n\sin\theta \qquad (1)$$

where n – refractive index of a media, θ – half-angle of a cone within which light rays converge or diverge. For a lens, this angle is defined by its diameter D and focal length F.

$$\sin\theta = \frac{D}{2F} = \frac{NA}{n} \qquad (2)$$

It is convenient to measure distances from the axis in the object plane by the units of the light wavelength in the media $\lambda' = \lambda/n$, where p – light wavelength in vacuum. Dimensionless radius unit in this case will be written as

$$p = \frac{2\pi}{\lambda} NAr = \frac{2\pi n}{\lambda} r\sin\theta \qquad (3)$$

while dimensionless distance ζ along the optical axis will be

$$\zeta = \frac{2\pi}{n\lambda} NA^2 z = \frac{2\pi n}{\lambda} z\sin^2\theta \qquad (4)$$

- What factors determine a microscope resolution?

Images are formed by lenses or mirrors in geometrically conjugate planes. In this case, for rays emanating from every point of the object, the Fraunhofer diffraction condition is met. Let, for example, a parallel beam from the distant point object converge in a lens focal plane (Fig. 3). Each point in the focal plane corresponds to the point at infinity, therefore, the Fraunhofer diffraction condition is met in the focal plane. Diaphragm D which confines the beam plays the role of an obstacle for light diffraction. Such a diaphragm, in particular, can be the lens mount. This is the case of the diffraction at the optical system entrance aperture.

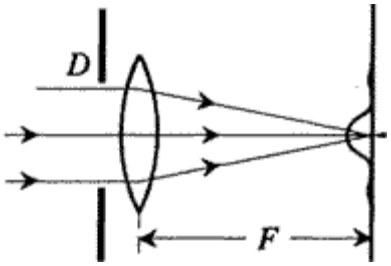

Fig. 3. Fraunhofer diffraction in a lens focal plane

Similarly can be considered the case when the point object is positioned at a finite distance a from the lens and the image is formed at a distance b from the lens on its right-hand side. Distances a and b obey the **lens formula**:

$$\frac{1}{a}+\frac{1}{b}=\frac{1}{F} \qquad (5)$$

To explain why Fraunhofer diffraction takes place in this case also, we replace the single lens with focal length F by two closely situated lenses with focal lengths F_1 and F_2 **(Fig. 4)**. Then the source will be positioned in the front focal point of the first lens and the image plane coincides with the rear focal plane of the second lens. Condition (1) is automatically met in this case because it is equivalent to the optical power (i.e. inverse of a focal length) sum rule of two closely situated lenses. Between the two lenses light rays travel as a parallel beam. Comparing **Fig. 3** and **Fig. 4** it can be concluded that in the second case the Fraunhofer diffraction occurs at the common lens mount and is viewed in the rear focal plane of the second lens. **Fig. 3** corresponds to the diffraction pattern in the telescope objective (or the eye), **Fig. 4** – to the light diffraction pattern in the microscope objective. Field of view of conventional microscopes does not exceed 1000 resolved picture elements.

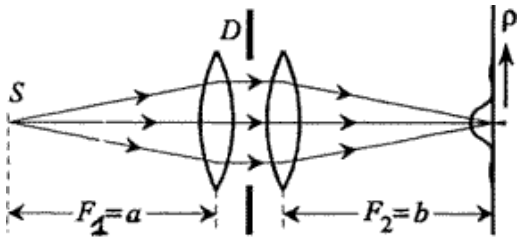

Fig. 4. Fraunhofer diffraction in a plane geometrically conjugate with a source

- Point spreading function (PSF)

The point spreading function (or the function of the diffraction-limited system pulse response) determines the intensity distribution in the lens focal plane due to the Fraunhofer diffraction from the entrance aperture. As it was shown before, exactly the same intensity distribution from a point source is formed in the conjugate plane of a thin lens.

PSF of the light beam limited by a circular aperture with diameter D for the lens having focal length F can be expressed in a general form as follows [2]:

$$p(\zeta,p)=|I_0(\zeta,p)|^2+2|I_1(\zeta,p)|^2+|I_2(\zeta,p)|^2 \qquad (6)$$

where

$$I_0(\zeta,p)\int_0^{\theta} J_0(p\sin\alpha/\sin\theta)\sqrt{\cos\alpha}\,\sin\alpha(1+\cos\alpha)\exp(i\zeta\cos\alpha/\sin^2\theta)d\alpha$$
$$I_1(\zeta,p)\int_0^{\theta} J_1(p\sin\alpha/\sin\theta)\sqrt{\cos\alpha}\,\sin^2\alpha\exp(i\zeta\cos\alpha/\sin^2\theta)d\alpha$$
$$I_2(\zeta,p)\int_0^{\theta} J_2(p\sin\alpha/\sin\theta)\sqrt{\cos\alpha}\,\sin\alpha(1-\cos\alpha)\exp(i\zeta\cos\alpha/\sin^2\theta)d\alpha \qquad (7)$$

where $J_k(x)$ – k-th order Bessel functions, $\sin\theta=\frac{D}{2F}=\frac{NA}{n}$.

Here we introduce the more general function as compared with that given before. Function $p(\zeta,p)$ gives intensity distribution along radius p for

different planes ζ. This function has a remarkable property for any plane ζ:

$$\int_0^\infty p(\zeta, p)\, pdp = const \qquad (8)$$

which means that energy flux through every plane is constant.

In the paraxial approximation (small *NA* magnitudes), the light intensity distribution in the focal plane is given by:

$$p(0, p) \approx \left(\frac{2J_1(0, p)}{p} \right)^2 \qquad (9)$$

where the normalization coefficient is selected so that $p(0,0)$ value in a focal point is equal to 1.

The diffraction pattern from a circular aperture is concentric rings. A central bright spot is called the **Airy disk**. The first bright ring maximum intensity is about 2% of the intensity in the center of the Airy disk. Distribution $p(0, p)$ is shown in **Fig. 5**.

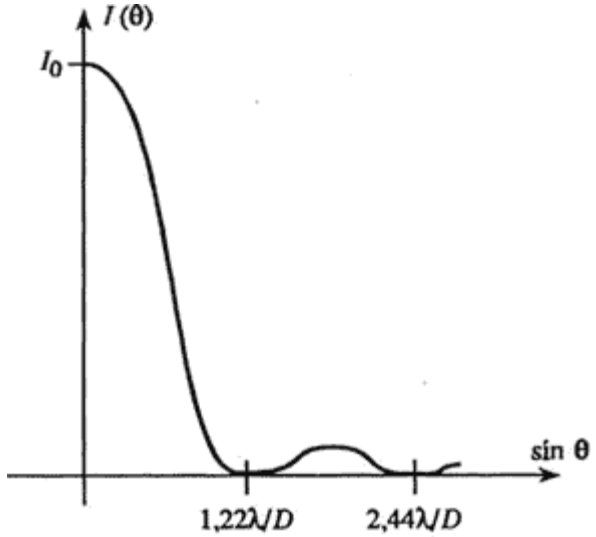

Fig. 5. Intensity distribution of light diffracted by a circular aperture

The Airy disk radius is:

$$p_{resel} = 1.22\pi \qquad (10)$$

or

$$r = 0.61\frac{\lambda}{n\sin} = 1.22\frac{\lambda'}{D}F \qquad (11)$$

where $\lambda' = \lambda / n$.

It should be noted that on the system optical axis $(p = 0)$: $I_1(\zeta,0) = 0$ and $I_2(\zeta,0) = 0$, therefore the resolution along the optical axis is determined only by contribution of $I_2(\zeta,0)$. In the paraxial approximation (small *NA* magnitudes), the relative intensity distribution along the axis is given by:

$$p(\zeta,0) \approx \left(\frac{\sin(\zeta/4)}{(\zeta/4)} \right)^2 \qquad (12)$$

- Microscope resolution, Rayleigh criterion.

Resolution of the microscope generally means the capability to distinguish two point objects of about equal intensity. From the function of intensity distribution in a focal plane $p(0, p)$ it follows that the resolution is determined by overlapping of Airy disks of two point-like objects. Rayleigh proposed the criterion which states that two points are resolved if a "dip" in their images intensity is 26% of the maximum intensity. Also, the separation distance between two resolved points should be more than the Airy disk radius (see previous paragraph).

Summary

- The main characteristic of the objective lens is its numeric aperture determined by its diameter and a focal length.
- Resolution of a conventional optical microscope is determined by the Fraunhofer diffraction at the entrance aperture of the objective lens. The minimum distance between resolved point objects of equal intensity amounts to the Airy disk radius.
- In present chapter, the expression for the point spreading function (or the function of the diffraction-limited system pulse response) is derived which will be used further for the explanation of the confocal microscope operation.

References

1. Robert H. Webb, "Confocal optical microscopy" Rep. Prog. Phys. 59 (1996) 427-471.
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3.2 Confocal Microscopy

- Introduction

A confocal microscope differs from a "classical" optical microscope (see chapter 3.1 "Classical" Optical Microscopy) in the fact that every moment of time there is formed an image of one object point while a whole image is assembled by scanning (moving a specimen or readjusting an optical system). In order to register light from only one point, a pinhole aperture is situated behind the objective lens so that the light emitted by the studied point (red rays in **Fig. 1b**) passes through the aperture and is detected while light from the other points (e.g. blue rays in **Fig. 1b**) is at most excluded. The second feature is that the illuminator produces not the uniform lighting of the field of view but focuses light into the studied point **(Fig. 1c)**. This can be done by placing a second focusing system behind a specimen; in this case, however, the specimen should be transparent. Moreover, the objective lenses are usually expensive enough, so utilization of a second focusing system for illumination is of little preference. An alternative is the use of a beam splitter for the purpose of incident and reflected light could be focused by the same objective **(Fig. 1d)**. Besides, such arrangement facilitates system adjustment.

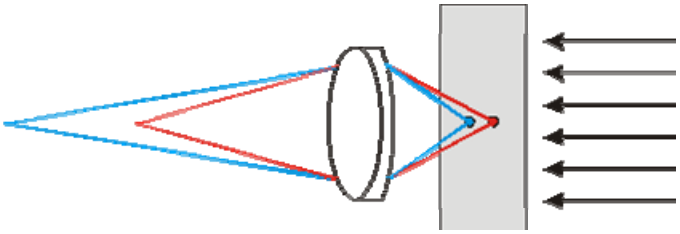

Fig. 1a. Traces of light rays in conventional microscope. The photodetector receives light from various points of a specimen

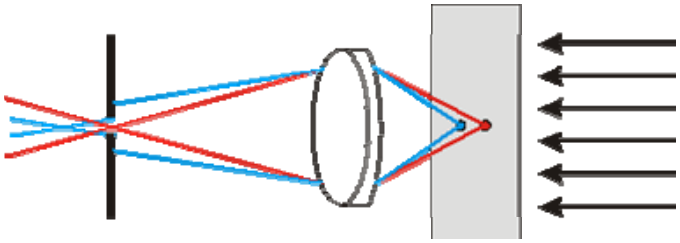

Fig. 1b. Aperture utilization allows to reduce sufficiently background illumination from specimen points beyond the studied area

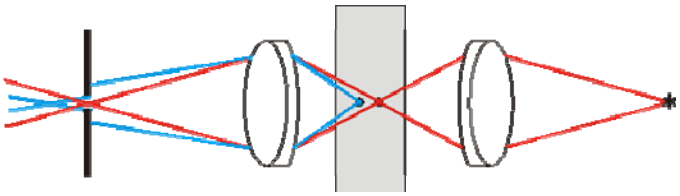

Fig. 1c. Additional contrast increase is due to illumination light focusing into the analyzable point

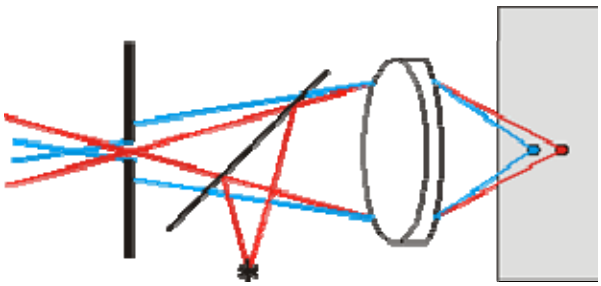

Fig. 1d. Arrangement with a beam splitter simplifies the microscope construction and facilitates its adjustment due to the objective two-fold use (for illumination and reflected light collection)

It is clear that the application of the confocal scheme should increase the image contrast because "stray" light from points adjacent to the studied one does not enter the detector. Note that the contrast increase is achieved at the expense of complicated scan by specimen or by light beam systems utilization. Detailed examination of existing confocal microscope designs is beyond the scope of this chapter. Further information on the matter can be found in reviews [1 - 11].

- Resolution and contrast in confocal microscopy

Now let us examine mathematically how and how much the contrast is changed when utilizing the confocal microscopy. Firstly, because the light in the confocal microscope passes through the objective twice, the point spreading function (designated further as **PSF**, see definition in chapter 3.1 "Classical" Optical Microscopy) is given by

$$p_{conf}(\zeta, p) = p(\zeta, p) \times p(\zeta, p) \qquad (1)$$

For the sake of convenience each PSF will be qualified as a probability of a photon hitting the point with coordinates (ζ, p) or a photon detection from the point with coordinates (ζ, p); then the confocal PSF is a product of independent probabilities. **Fig. 2** shows a representation of conventional and confocal PSF.

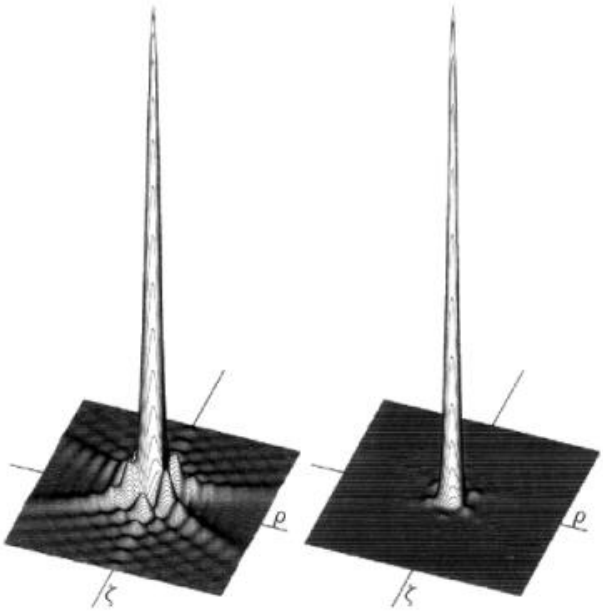

Fig. 2. Confocal PSF $p_{conf}(\zeta, p) = p(\zeta, p) \times p(\zeta, p)$ is shown on the right, conventional PSF (ζ, p) – on the left

If we use the Rayleigh criterion for the resolution (26% dip of the maximum intensity), the result is a slight increase in resolution for the confocal microscope:

$$r_{conf} = 0.44\frac{\lambda}{n\sin\theta} = 0.88\frac{\lambda'}{D}F \qquad (2)$$

as compared with the conventional optical microscope

$$r_{conf} = 0.61\frac{\lambda}{n\sin\theta} = 1.22\frac{\lambda'}{D}F \qquad (3)$$

where $\lambda' = \lambda/n$.

However, the major advantage of a confocal microscope is a sufficient increase in the contrast rather than resolution improvement in accordance with the Rayleigh criterion. In particular, the relation of the first ring maximum amplitude to the amplitude in the center is 2% in case of conventional PSF in a focal plane while in case of a confocal microscope this relation is 0.04%. The practical importance of this factor is illustrated in **Fig. 3**. From the top part of the picture it can be seen that a dim object (intensity 200 times less than of a bright one) can not be detected in conventional microscope though the separation distance between objects exceeds that of the Rayleigh criterion. In a confocal microscope (bottom part of Fig. 3) this object should be well registered.

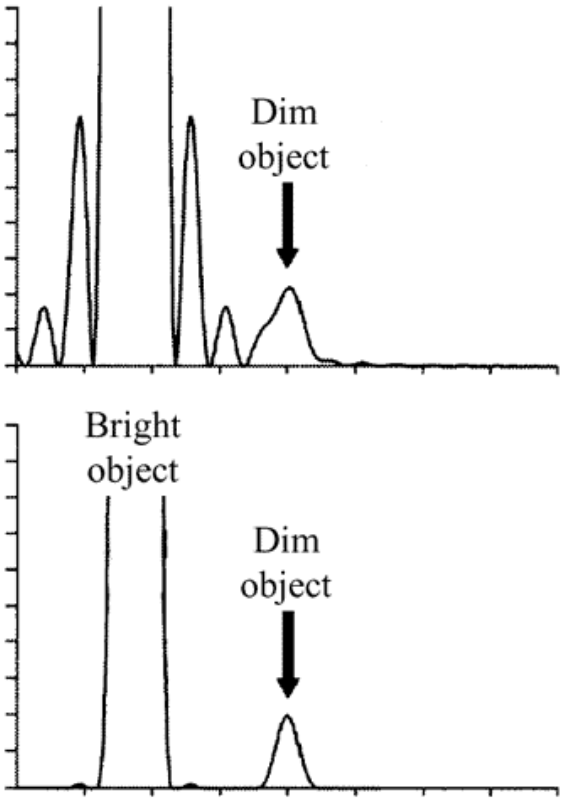

Fig. 3. Intensity profiles for conventional (top picture) and confocal (bottom picture) microscopes. Intensity maximum of the dim object is 200 times less than that of the bright one

The intensity distribution along the optical axis in a confocal microscope is given by the following expression:

$$p_{conf}(\zeta,0) \approx \left(\frac{\sin(\zeta/4)}{\zeta/4}\right)^4 \qquad (4)$$

Then, using the Rayleigh criterion for the resolution in the direction along the optical axis we can write:

$$\Delta z_{conf} = 1.5\frac{\lambda}{n\sin^2\theta} = 1.5\frac{n\lambda}{NA^2} = 6\lambda'\left(\frac{F}{D}\right)^2 \qquad (5)$$

Notice that one should distinguish this resolution and depth of focus in a conventional microscope. Generally, the depth of focus is hundreds times more than the resolution along the optical axis.

- The effect of an aperture in a focal plane

One of parameters, that was not taken into consideration above, is the size of an aperture in a focal plane of illuminating and collecting lenses. Notice that PSF for conventional and confocal microscopes was calculated under assumption that a source is point-like. Therefore the obtained PSF describe properties of an objective lens and the aperture image in the object plane determines areas whose light is registered by the photodetector. Lowering the aperture size obviously decreases the amount of the passing light, increases noise level and, finally, can reduce all advantages in the contrast to nothing. Thus, the problem of choosing the aperture optimal size and reasonable compromise is quite relevant.

The use of aperture with the size which is less than that of the Airy disk just results in intensity loss and do not affect resolution. If aperture size is that of the Airy disk, the objective lens resolution is maximal. The best compromise, however, is the aperture size which is 3-5 times more than that of the Airy disk. The size considered here should be understood as the image size in the object plane, therefore, the actual aperture size depends on the lens magnification. In particular, when lens with 100x magnification is used, the 1 mm orifice of the diaphragm is projected onto the object plane as a circle with radius 10 micron.

In order to consider mathematically the presence of an aperture and to obtain a new function of intensity distribution one should perform convolution:

$$P(p,\zeta) = p \otimes S = \int p(p-p_s,\zeta)S(p_s,\varphi_s)p_s d\varphi_s dp_s \qquad (6)$$

and for a confocal microscope to multiply the obtained function $P(p,\zeta)$ by $p(p,\zeta)$. The resulting intensity distribution in case of the aperture size of 5 Airy disks is shown in **Fig. 4.**

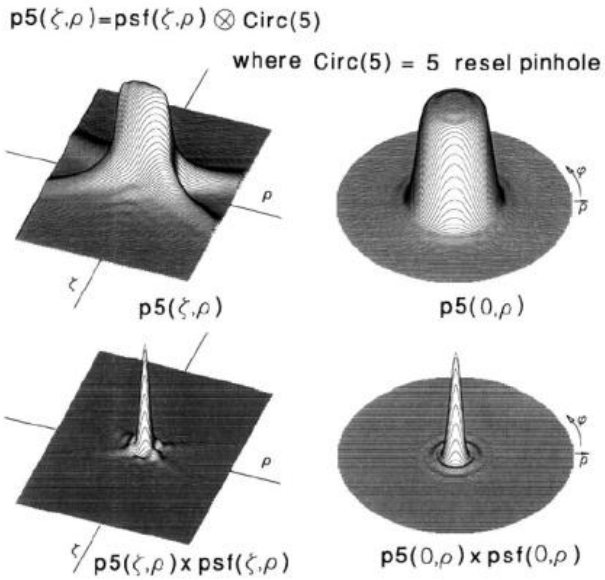

Fig. 4. Point spreading functions for conventional microscope with an aperture size of 5 Airy disks (top pictures) and for confocal microscope (bottom pictures)

Summary

- Confocal microscopy provides an image contrast increase due to the studied area illumination via focusing objective lens and an aperture placement in the image plane before the photodetector. Such contrast improvement allows for resolving objects having intensity difference up to 200:1.
- In confocal microscopy, the resolution in the object plane is slightly increased (1.5 times) while the resolution along the optical axis is high.
- These improvements are obtained at the expense of the utilization of mechanisms for scanning either by moving a specimen or by readjustment of an optical system. Scanning application allows to increase field of view as compared with conventional microscopes.

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