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# Microscopes — Confocal microscopes — Optical data of fluorescence confocal microscopes for biological imaging

*Microscopes — Microscopes confocaux — Données optiques des microscopes confocaux à fluorescence pour l'imagerie biologique*



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# Foreword

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This document was prepared by Technical Committee ISO/TC 172 *Optics and photonics*, Subcommittee SC 5 *Microscopes and endoscopes*.



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# Introduction

This document is intended to provide comparable specifications of confocal microscopes by microscope manufacturers and to allow users to compare and monitor the imaging performance of their confocal microscopes.

A confocal laser scanning microscope in this document comprises a laser illumination light source, a scanning unit to deflect the excitation laser light, an objective and a detection unit consisting of a detection pinhole and a photo detector.

# Microscopes — Confocal microscopes — Optical data of fluorescence confocal microscopes for biological imaging

## 1 Scope

This document specifies commonly used quantities regarding image performance in confocal laser scanning microscopy used for imaging of fluorescent biological specimens.

This document applies only to confocal single point scanners using single photon excitation.

## 2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 10934-1, *Optics and optical instruments — Vocabulary for microscopy — Part 1: Light microscopy*

ISO 10934-2, *Optics and optical instruments — Vocabulary for microscopy — Part 2: Advanced techniques in light microscopy*



## 3 Terms and definitions



For the purposes of this document, the terms and definitions given in ISO 10934-1, ISO 10934-2 and the following apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at http://www.electropedia.org/

**3.1**
**excitation wavelength**
specific wavelength of light required to excite a fluorescent molecule, such as a fluorescent antibody or fluorescent protein, to emit light at emission wavelengths

**3.2**
**detection wavelength band**
specific wavelength range of light collected by the photo detector

**3.3**
**Airy unit**
**AU**
diameter of the theoretical first minimum of the detection PSF in the low numerical aperture approximation

$$AU = 1,22\frac{\lambda_{ref}}{NA}$$

where

NA is the numerical aperture;

$\lambda_{ref}$ is the reference wavelength.

**3.4**
**pixel**
smallest element of the digital image to which attributes are assigned

**3.5**
**pixel size**
shortest distance from the centre of one pixel to the centre of an adjacent pixel measured in object space

**3.6**
**confocal point spread function**
**cPSF**
product of the intensity point spread functions of the illuminating and detecting optical systems

[SOURCE: ISO 10934-2:2007 2.11.7, modified — the word "intensity" has been added, and "in a confocal microscope" has been removed from the definition.]



**3.7**
**coordinate system**
right-handed Cartesian coordinate system defined by the optical axis as z-axis and the x-y plane perpendicular to it



Note 1 to entry: The x and y coordinates are referred to as lateral coordinates, while z coordinates are referred to as axial coordinates.

**3.8**
**signal-to-noise ratio**
ratio of signal to its noise

**3.9**
**signal-to-background ratio**
ratio of signal to the background

# 4 Quantities

## 4.1 Resolution and strength of optical sectioning

### 4.1.1 General

Resolution is the capacity for imaging fine detail which is determined by the minimum spatial separation of two-point objects required for their observation as distinct objects. Various criteria have been proposed to determine the resolution, e.g. the Abbe, Rayleigh, Schuster, Houston or Sparrow criteria. In confocal microscopy, the resolution is commonly described by the full width at half maximum (FWHM) of the confocal point spread function (cPSF).

In general, the minimum resolvable distance is the most relevant quantity regarding resolution. For practical reasons this document defines resolution as given in 4.1.2.

In practice, other factors such as noise and signal background, as well as the FWHM of the cPSF, affect the minimum resolvable distance.

NOTE The term resolution is taken to refer to spatial, as opposed to temporal or spectral, resolution throughout this document.

### 4.1.2 Definition of resolution

Resolution is defined as the full width at half maximum (FWHM) of the cPSF measured in the centre of the object field.

The lateral resolution is given by the FWHM of the intensity signal along a lateral direction through the centre of a fluorescent point-like object.

The axial resolution is given by the FWHM of the intensity signal along the axial direction through the centre of a fluorescent point-like object.

### 4.1.3 Definition of strength of optical sectioning

The usefulness of confocal fluorescence microscopy is based to a large extent on the ability to suppress out-of-focus light and thereby enable optical sectioning of the specimen. The strength of optical sectioning is dependent on the spatial frequencies of the object scanned through the focus. A uniform fluorescent planar object is widely used and generally accepted as a test object for optical sectioning[3]. Another useful depth discrimination criterion is the evaluation of the detected signal of a mirror scanned through the focus[4]. While using a thin uniform fluorescent layer as the test object is closer to the considered application of the confocal microscope, measurement of a reflective planar object is easier to implement. Both the thin uniform fluorescent layer and the reflective planar object represent a step in the axial direction and therefore contain all spatial frequencies in the axial direction. Therefore, the strength of optical sectioning is defined as the FWHM of the signal of a planar object scanned through the focus as measured in the centre of the object field.



### 4.1.4 Measurement

The resolution is determined by imaging a small point-like object, e.g. a fluorescent microsphere. The point-like object shall be sufficiently smaller than the expected resolution, i.e. the diameter of the object should be smaller than half of the expected resolution. For the measurements of the resolution with objectives designed for use with cover glass, fluorescent objects which are mounted as close as possible to the cover glass should be chosen in order to minimize aberrations caused by the mounting medium. The theoretical resolution, computed for an ideal confocal fluorescence microscope, is listed in Annex A. In practice, the theoretical resolution is not achieved.

The strength of optical sectioning is determined as the FWHM of the signal of a thin uniform fluorescent layer or reflective planar object scanned through the focus. The signal is measured in the centre of the object field. The thin uniform fluorescent layer used for the measurement of the strength of optical sectioning with objectives designed for use with cover glass should be located preferably on the cover glass in order to minimize aberrations caused by the mounting medium. The thin uniform fluorescent layer shall be significantly thinner than the expected strength of optical sectioning, i.e. the thickness of the thin uniform fluorescent layer should be smaller than half of the expected strength of optical sectioning.

For detection pinholes differing from a circular pinhole, the characteristic size of the pinhole determining the resolution shall be identical to the diameter of a corresponding circular pinhole. The largest dimension of such a pinhole shall not be more than 50 % longer than the smallest dimension of the pinhole, e.g. the diagonal of a square pinhole is 41 % longer than its side.

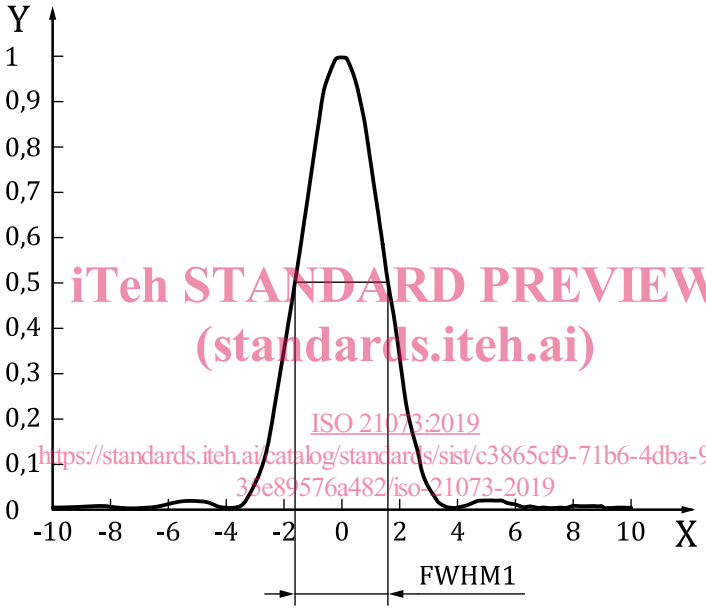
NOTE 1 For highest resolution, the detection pinhole size theoretically needs to be infinitesimally small. In order to obtain higher signal levels, the pinhole is opened up and commonly set to a diameter of 1 AU in fluorescence microscopy. Although the lateral resolution achieved with a pinhole diameter of 1 AU is only slightly better than the resolution of a widefield microscope, the confocal microscope still exhibits axial sectioning capability.

The pixel size shall be sufficiently smaller than the theoretical resolution, i.e. the pixel size should be at least 10-fold smaller than the theoretical resolution. The scanned field shall be at least 10-fold larger than the theoretical resolution. In the presence of side lobes greater than 50 % of the intensity maximum of the cPSF, a resolution shall not be stated, since the FWHM of the cPSF is ambiguous.

NOTE 2 Diffraction-limited imaging performance of a confocal microscope results in sidelobes much lower than 50 % of the intensity maximum of the cPSF.

Attention should be given to the signal-to-noise ratio when determining the FWHM of the cPSF. If the FWHM of the cPSF cannot be clearly determined from a single measurement, sufficient multiple measurements of one point-like object or multiple point-like objects shall be averaged.

In the recording of the cPSF the maximum and the minimum of the cPSF shall be included to avoid an incorrect determination of the FWHM. This is illustrated in Figure 1 and Figure 2, where clipping of the cPSF at a value of 0,2 results in a FWHM value FWHM2, which is about 14 % smaller than the correct FWHM value of FWHM1.



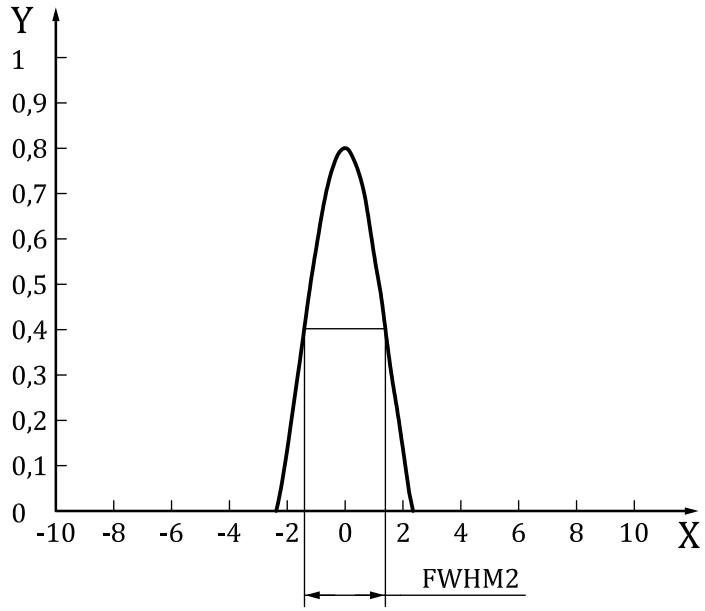



**Key**

X distance from centre [a.u.]

Y intensity [a.u.]

**Figure 1 — Determination of the correct FWHM value FWHM1 of the cPSF**




**Key**

X distance from centre [a.u.]

Y intensity [a.u.]

**Figure 2 — Clipping of the cPSF results in an incorrect FWHM value of FWHM2**



The FWHM shall be determined from the unprocessed recorded data, e.g. the data shall not be deconvolved. Further, the signal transmission path shall be linear, e.g. the excitation of the fluorophore and the signal from the detector shall not be saturated. A linear signal transmission path also involves the signal transmission path in the confocal microscope system, especially the electronics and the software.



The FWHM value shall be obtained by fitting a Gaussian function of the form

$$I(x) = A \cdot e^{-\frac{1}{2}\left(\frac{x-x_0}{\sigma}\right)^2} + c$$

to the measured data, where $A$, $x_0$, $\sigma$ and $c$ are subject to be fitted. The FWHM is given by

$$\text{FWHM} = 2 \cdot \sqrt{2 \cdot \ln(2)} \cdot \sigma \approx 2{,}35 \cdot \sigma$$

The Gaussian fitting shall be performed from the intensity profiles of a line, i.e. a width of one pixel.

The measurement of the resolution and the strength of optical sectioning is strongly dependent on the following parameters, which shall be declared together with the resolution/strength of optical sectioning statement:

— Excitation wavelength;

— Detection wavelength band;

— Manufacturer's designation of the objective;

— Size of detection pinhole in Airy units and reference wavelength used to specify the Airy unit. In general a detection pinhole size of 1 Airy unit should be used;

— Polarization state and, in the case of linear polarized excitation light the direction of polarization with respect to the axis for which the lateral resolution is measured (see NOTE 3);