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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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ISO copyright office
CP 401 • Ch. de Blandonnet 8
CH-1214 Vernier, Geneva
Phone: +41 22 749 01 11
Fax: +41 22 749 09 47
Email: copyright@iso.org
Website: www.iso.org

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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

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;

λ_{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.

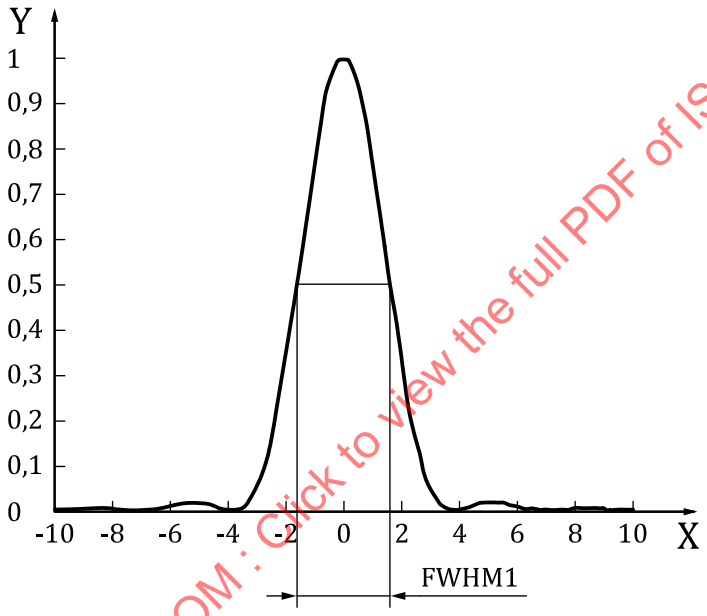
NOTE 1 For highest resolution, the detection pinhole size theoretically needs to be infinitesimally small. In order to obtain higher signal levels, the pinhole is opened up and commonly set to a diameter of 1 AU in fluorescence microscopy. Although the lateral resolution achieved with a pinhole diameter 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.

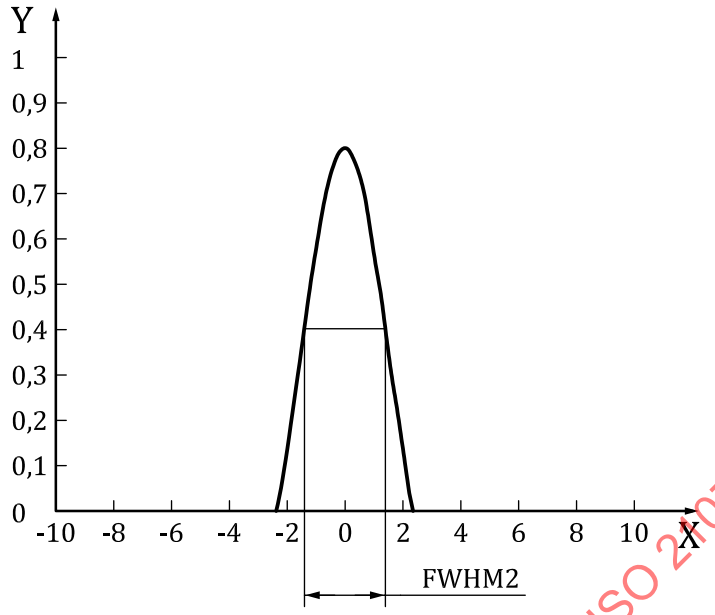


Key

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, σ 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);

— Diameter of the fluorescent point-like object used for the measurements;

— Type of planar object, i.e. reflective or fluorescent, used for the measurement of the strength of optical sectioning;

— Distribution of laser illumination in the objective pupil; the distribution of the laser illumination should be uniform or a Gaussian distribution and not a distribution of special shape, e.g. annular illumination;

— The correlation coefficient as a measure of the goodness of fit.

In order to determine the correct values for resolution and strength of optical sectioning, the instructions of the manufacturer shall be met. This applies especially to correct immersion medium, mounting medium or imaging buffer, cover glass type and cover glass thickness, temperature and orientation of specimen. If the objective is fitted with a correction collar, it is important that it is set to the correct position.

NOTE 3 For high numerical aperture objectives and linear polarized excitation light, the FWHM in the direction perpendicular to the direction of the polarization is smaller than the FWHM in the direction of the polarization.

4.2 Uniformity of field and centring accuracy

4.2.1 Definition of uniformity of field and centring accuracy

In order to allow the investigation of the object, the confocal microscope should achieve a certain degree of image uniformity. Otherwise details of the object might not be detected.

The uniformity of the brightness in the image field is expressed as

$$uniformity\,[\%] = 100 \times \frac{\text{minimum brightness in the image field}}{\text{maximum brightness in the image field}}$$

The centring of the brightness maximum in the image is expressed as the centring accuracy CA and is given by the following formula:

$$CA\,[\%] = 100 - 100 \times \sqrt{\left(x_{\max} - x_{\text{centre}}\right)^2 + \left(y_{\max} - y_{\text{centre}}\right)^2} \times \frac{2}{\sqrt{w^2 + h^2}}$$

where

$\{x_{\max}, y_{\max}\}$ are the coordinates of the brightness maximum;

$\{x_{\text{centre}}, y_{\text{centre}}\}$ are the coordinates of the centre of the field;

w and h are the width and height of the field, respectively.

4.2.2 Measurement

The measurements of uniformity and centring accuracy shall be performed with a homogeneous fluorescent specimen, e.g. a solution of a fluorescent marker. Solutions of markers are generally preferable over solid samples, since bleached fluorophores in the image field are replaced by unbleached fluorophores due to diffusion.

The pinhole diameter shall be set to the same value as was used for the resolution measurements. The minimum number of pixels used for the measurement shall be 128 pixels × 128 pixels.

While performing the measurement, it has to be ensured that the complete data recorded originates from the homogeneous fluorescent region of the specimen to avoid misjudgement, which might be caused, e.g. by field of curvature of the optics or a tilted specimen. The signal-to-noise ratio has to be

high enough to clearly determine the uniformity and centring accuracy. The signal-to-noise ratio is high enough if the uniformity varies less than 5 percentage points when the illumination intensity is changed by a factor of at least 1,5. 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.

If information on image uniformity and/or centring accuracy is provided to the user, this information shall be given according to the definitions given in 4.2.1 and contains the following additional data:

— Field number of the confocal scan optic (see 4.5) and percentage of field number, which corresponds to the scanned field of the measurement;

— Information on the excitation wavelength;

— Information on the 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 order to determine the correct values for uniformity of field and centring accuracy the instructions of the manufacturer shall be met. This applies especially to correct immersion medium, cover glass type and cover glass thickness, temperature.

4.3 Co-registration accuracy

4.3.1 Definition of co-registration accuracy

Co-registration accuracy is the precision with which a confocal microscope system images a fluorescent object with different excitation and emission wavelengths at the same position in the image. Hence, the spatial distance of the image positions of the fluorescent object located in the centre of the object field for different excitation and detection wavelength bands is denoted as co-registration accuracy. This differs from the fact that for different wavelengths the lateral position of an object in the image can vary over the image field due to the lateral chromatic aberration of the optical system. The co-registration accuracy shall be calculated from the intensity maxima or the intensity centre of gravity of the image of the object for the different wavelengths.

The lateral co-registration accuracy is expressed as

$$\Delta_{\text{lateral}} = \sqrt{\left(x_{\lambda 1} - x_{\lambda 2}\right)^2 + \left(y_{\lambda 1} - y_{\lambda 2}\right)^2}$$

and the axial co-registration accuracy is expressed as

$$\Delta_{\text{axial}} = \sqrt{\left(z_{\lambda 1} - z_{\lambda 2}\right)^2}$$

where

$x_{\lambda 1}$ is the x-coordinate of the image position of the object for the detection wavelength band λ_1;

$y_{\lambda 1}$ is the y-coordinate of the image position of the object for the detection wavelength band λ_1;

$z_{\lambda 1}$ is the z-coordinate of the image position of the object for the detection wavelength band λ_1;

$x_{\lambda 2}$ is the x–coordinate of the image position of the object for the detection wavelength band λ_2;

$y_{\lambda 2}$ is the y-coordinate of the image position of the object for the detection wavelength band λ_2;

$z_{\lambda 2}$ is the z-coordinate of the image position of the object for the detection wavelength band λ_2.

4.3.2 Measurement of co-registration accuracy

The measurement of co-registration accuracy shall be performed with objects labelled with fluorescent markers emitting over a wide wavelength range or multiple wavelength bands. The objects shall be located in the centre of the object field and shall be small in size compared to the resolution or have the same distribution density for different fluorescent markers over their volume. Homogeneously fluorescent objects yielding multiple excitation and fluorescent bands are suitable objects for co-registration measurements.

The objects used for the measurements with objectives designed for use with cover glasses should be close to the cover glass to avoid aberrations caused by the mounting medium. The object shall also be in the centre of the object field. The pixel size shall be sufficiently smaller than the expected resolution of the objective. The pixel size should be at least 10-fold smaller than the expected resolution of the objective.

A three-dimensional data stack (x, y, z) enclosing the object shall be recorded. The positions of the object for different fluorescent bands shall be determined by calculating the intensity centre of gravity or by calculating the maxima by interpolation in case the object structure is known, e.g. as is the case for a fluorescent microsphere.

If the position of the object cannot be clearly determined from a single measurement, sufficient multiple measurements of one object shall be averaged.

If information on co-registration accuracy is provided to the user, this information shall be given according to the definitions given in 4.3.1 and contains the following additional data:

— Information on the excitation wavelengths;

— Information on the detection wavelength bands;

— Manufacturer's designation of the objective;

— Size of detection pinhole in Airy units and reference wavelength used to specify the Airy unit;

— Information about detection arrangement, e.g. single detector for all wavelength bands or individual detector for each wavelength band;

— Information whether the intensity centre of gravity of the object or the maximum of the cPSF was used.

In order to determine the correct values for the co-registration, the instructions of the manufacturer shall be met. This applies especially to correct immersion medium, mounting medium or imaging buffer, cover glass type and cover glass thickness, temperature and orientation of specimen.

4.4 Stability of illumination power

4.4.1 General

For confocal imaging the stability of the illumination power is of high importance. In this document the changes in the illumination power, which are on a time scale of the acquisition time of 2-dimensional images or 3-dimensional image stacks are considered. These durations are on a much longer time scale than the pixel dwell time and therefore do not include noise effects such as laser noise. The stability is the percentage deviation of minimum and maximum illumination powers from the average power during the time interval considered.

4.4.2 Measurement of stability of illumination power

The measurement is performed with a 10× objective and a calibrated power meter placed so that the detection area of the power meter is slightly underfilled with light. The detection range of the power meter shall be in the linear range. If a 10× objective is not available, the measurement shall be performed without an objective and the power meter shall be placed at the position of the pupil of the objective. Thus the objective needs to be removed. The power meter shall be mounted in a fixed position in regard to the objective or the objective turret respectively. External influences on the measurement, e.g. changes in room temperature or changes in the lightning conditions, have to be eliminated. The lasers shall be warmed-up according to the manufacture's specifications. If the manufacturer does not specify a warm-up time, the lasers shall be warmed-up for at least one hour prior to measurement. The light output of the lasers should be set to a power value, which results in a power reading of 0,2 mW to 0,5 mW on the detector of the power meter. During the actual recording of the laser power, the illumination beam shall be stationary and the laser light shall continuously illuminate the sensor of the power meter, i.e. no blanking of the laser light. The detector of the power meter shall not be saturated. The integration time of the power meter shall be between 0,2 s and 1 s.

In order to determine the stability of the illumination power, the following measurements are conducted:

a) Short term stability of illumination power

The laser power shall be recorded for a time interval of 5 min. During this time interval the laser light is continuously illuminating the detector of the power meter and a power reading is recorded every second. The short term stability of illumination power ($STAB_{short}$) is expressed as

$$STAB_{short}\ [\%] = 100 \times \left(1 - \frac{P_{max} - P_{min}}{P_{max} + P_{min}} \right)$$

Where

P_{max} is the maximum laser power recorded during the time interval of 5 min;

P_{min} is the minimum laser power recorded during the time interval of 5 min.

b) Long term stability of illumination power

The laser power shall be recorded for a time interval of 120 min. During this time interval the laser light is illuminating the detector of the power meter every 30 s for one second. During the one second illumination of the detector a power reading is recorded. The long term stability of illumination power $STAB_{long}$ is expressed as

$$STAB_{long}\ [\%] = 100 \times \left(1 - \frac{P_{max} - P_{min}}{P_{max} + P_{min}} \right)$$

where

P_{max} is the maximum laser power recorded during the time interval of 120 min;

P_{min} is the minimum laser power recorded during the time interval of 120 min.

NOTE In contrast to these definitions some laser manufacturers define "stability" as fluctuation of laser power.

If information on the short or long term stability of illumination power is provided to the user, this information shall be given according to the definitions given in 4.4.2 and contain the following additional data:

— Information on the laser wavelengths, which the stability is given for;

— Information on the average laser power measured.

4.5 Field number of the confocal scan optic

4.5.1 General

The size of the scanned field is important for imaging specimens in a confocal laser scanning microscope, because larger fields enable images of larger objects. The scanned field depends on the field number of the confocal scan optics and the magnification of the objective. Hence, the field number of the confocal scan optics is a suitable quantity to describe the size of field of a confocal scanning microscope, since it is independent of the objective.

4.5.2 Definition of field number of the confocal scan optic

The field number of the confocal scan optic is expressed as max. diameter of scanned field [mm] × magnification of objective.

The maximum diameter of the scanned field and the field number of the confocal scan optic are defined for single images, i.e. without shifting the object and stitching single images into a total larger image.

Typically the images have a square format. Hence, the diameter of the scanned field is $\sqrt{2}$ times the length of one edge of the scanned field.

4.5.3 Measurement of maximum diameter of scanned field

In order to determine the field number of the confocal scan optic, the magnification of the objective as specified by the objective manufacturer is used for the calculation. A test specimen with an object structure of known size (e.g. a grid structure, a fluorescent target structure, or a matrix of fluorescent micro-objects) is imaged using the objective and the maximum scanned field possible. Imaging of the test specimen may be conducted with transmitted light. In order to determine the correct values for the maximum diameter of the scanned field, the instructions of the manufacturer for the objective shall be met. This applies especially to correct immersion medium, mounting medium or imaging buffer, cover glass type and cover glass thickness, temperature and orientation of specimen.

The maximum diameter of the scanned field $d_{\text{scan field}}$ is expressed as

$$d_{\text{scan field}} = \frac{S_{\text{object}} \times d_{\text{image}}}{S_{\text{image}}}$$

where S_{object} is the known size of the object structure, d_{image} is the maximum diameter of the image and S_{image} is the measured size of the image of the object structure.

NOTE 1 The actual size of the scanned field depends on the field number of the confocal scan optic and the deflection range of the scanning mirrors (zoom value) set in the microscope controls. Confocal laser scanning microscope manufacturers use different definitions for the zoom values used in their microscope controls. Hence, the zoom values are inappropriate to compare different microscopes.

Measurements with different objectives can lead to slightly different values for the field number of the confocal scan optic due to magnification tolerances of the objectives according to ISO 8039.

4.6 Scanning frequency

Image acquisition time is a crucial property of a confocal laser scanning microscope. Especially when imaging objects in motion as is the case in live cell imaging, the scanning speed can determine the success of the experiment.

The scanning frequency is dependent on the scanned format (line, frame or stack) and expressed as

$$f_{\text{line}} = \frac{N_{\text{line}}}{t}$$

or

$$f_{\text{frame}} = \frac{N_{\text{frame}}}{t}$$

or

$$f_{\text{stack}} = \frac{N_{\text{stack}}}{t}$$

where

f_{line} is the line scanning frequency in Hz;

f_{frame} is the frame scanning frequency in Hz;

f_{stack} is the stack scanning frequency in Hz;

N_{line} is the number of lines;

N_{frame} is the number of frames;

N_{stack} is the number of stacks;

t is the time in seconds required to acquire the lines, frames or stacks including the time required for repositioning the scanning device to the starting point.

The scanning frequency depends on whether image data are acquired in unidirectional or bidirectional scan mode. In unidirectional scan mode, data are only acquired while the laser beam moves in one direction. No data are recorded while the laser beam is being guided to the starting point of the next line. In bidirectional scan mode, one line is scanned in one direction, e.g. from left to right, and the next line in the opposite direction, i.e. from right to left. In other words, the return run, or flyback, of the laser beam is used for recording data as well, thus increasing the scanning frequency when using bidirectional scan mode.

In bidirectional scan mode, special care shall be taken that the phase differences between the forward sweep and the flyback are adjusted correctly. Otherwise the pixels of the forward sweep and flyback are not aligned to each other.

The scanning frequencies shall be given for continuous data transfer of the image acquisition system to the data storage system. The scanning frequency for a frame f_{frame} should be given for a frame size of 512 pixels × 512 pixels.

If information on the scanning speed is provided to the user, this information shall be given as line scanning frequency, frame scanning frequency or stack scanning frequency according to the definitions given above and contain the following additional data:

— Information on the size of the frame, i.e. number of pixels in the lateral directions;

— Information on the size of the stack, i.e. number of pixels in the lateral directions and in the axial direction;

— Field number of the confocal scan optic for which the scanning frequency is given;

— Information on the number of acquisition channels;