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Surface chemical analysis — Surface characterization — Measurement of the lateral resolution of a confocal fluorescence microscope

Analyse chimique des surfaces — Caractérisation des surfaces — Mesurage de la résolution latérale d'un microscope confocal à fluorescence

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

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Foreword

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The committee responsible for this document is ISO/TC 201 *Surface chemical analysis*.

Introduction

Confocal fluorescence microscopes (CFMs) are laser scanning confocal microscopes (LSCMs) operated in a fluorescence imaging mode so as to obtain a fluorescence image of a sample. Fluorescence is the light emitted by a molecule or solid lattice during relaxation after undergoing photon absorption and electronic excitation. The fluorescence wavelength, intensity and spectral shape are specific to the electronic structure of the material; therefore, fluorescence spectroscopy and imaging techniques are useful for chemical characterization and analysis. Among the optical imaging and spectroscopy tools, CFM yields a high spatial resolution that is advantageous for analysing nanomaterials and thin films. The spatial resolution is one of the most important performance factors for a CFM.

The spatial resolution of a technique refers to the maximum resolvability of two adjacent objects. This value is often characterized in different ways by the manufacturers. The spatial resolution of a CFM is characterized by both the lateral and axial resolution, which have different values and are not necessarily dependent on one another. In this International Standard, one convenient and effective method for measuring the lateral resolution of a CFM is presented. This method is suitable for use by non-expert operators.

Surface chemical analysis — Surface characterization — Measurement of the lateral resolution of a confocal fluorescence microscope

1 Scope

This International Standard describes a method for determining the lateral resolution of a confocal fluorescence microscope (CFM) by imaging an object with a size much smaller than the expected resolution.

2 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

2.1
point spread function
response of an imaging system to a point source or point object

2.2
lateral resolution
distance measured either in the plane of the sample surface or in a plane at right angles to the axis of the image forming optics over which changes in composition can be separately established with confidence

Note 1 to entry: See Reference[1].

3 Symbols and abbreviated terms

OL	objective lens
APD	avalanche photodiode
FWHM	full width at half-maximum
CFM	confocal fluorescence microscope
NA	numerical aperture
PSF	point spread function
QD	quantum dot
PMT	photomultiplier tube

4 General

4.1 Background information

Laser scanning confocal microscopes (LSCMs) scan a tightly focused laser beam over a sample and record the optical intensity at each pixel to form a two-dimensional image. LSCMs have a pinhole in front of the photo detector or a spectrometer input slit at the conjugate focal plane of the laser focus at the sample. Light originating from the non-focal plane is largely prevented from reaching the photo detector. Confocal configurations significantly improve the contrast in an image, and the spatial resolution may be increased[2].

CFM is one of the most widely used LSCM operation modes because it provides a fluorescence image or spectrum. In the fluorescence mode, the incident laser light is blocked by a long pass filter, and only the Stokes-shifted fluorescence light is detected by the photo detector or spectrometer. An image is formed, depending on the fluorescence wavelength, and the image displays good contrast compared to images obtained through other optical imaging techniques. Imaging multiple colours in one image is also possible.

The spatial resolution is one of the most important features characterizing the performance of an LSCM or CFM. The lateral resolution and axial resolution must be determined separately and must be treated independently. The lateral resolution is important especially when a CFM is used for the imaging and chemical analysis of thin films or nanoscale objects, in which the axial dimension is significantly less than the typical value of the axial resolution of a CFM.

The spatial resolutions of instruments tend to be characterized in different ways by different manufacturers. This work provides one convenient and effective method for measuring the lateral spatial resolution of a CFM instrument so as to be suitable for use by a non-expert operator. The terms and analysis procedure described here are according to ISO 18516[3].

4.2 Types of CFM operation

4.2.1 General

Below, we describe different modes of CFM operation according to the laser focus scanning technique with respect to the sample. This International Standard principally treats stage scanning-type and laser scanning-type CFMs.

4.2.2 Stage scanning CFM

Stage scanning CFMs are characterized by a moving stage that implements a scanning function while the laser focus remains unmoved with respect to the microscope frame. Because the optics involved in the CFM are stationary, the beam path is simple and nearly maintenance-free. Aberrations or drift in the optical alignment are minimized. The scan area size is limited only by the mechanical movement of the sample scanning stage, and allows for large-area scans.

Scanning speeds in these CFMs are relatively slow, and the sample may be affected by any rapid movements of a scanning stage and, therefore, may not be suitable for imaging delicate cells.

4.2.3 Laser scanning CFM

Off-axis beam scanning techniques can incur aberrations that degrade the image resolution. A fast oscillating mirror set may be used to scan a beam across a sample more rapidly than is possible in stage scanning-type CFM. The scanning rate can sometimes reach values of a few kHz line scan speed. In this approach, the sample does not move, thereby preserving the condition of the sample.

Because the optical ray is off-axis during laser focus scanning, some aberrations may be introduced into the image. The need for a scanning head makes this technique more complicated than techniques based on stage scanning. The size of a scan area is limited because an objective will admit off-axis laser light within only limited angle. The size of a scanning area also depends on the magnification of the objective. The use of low magnification objectives (resulting in a large scan area) which at the same time have a high NA (which results in a good collection efficiency and an improved resolution) is preferential in this case.

4.2.4 Spinning disk CFM

High-speed spinning disks involve multiple laser foci and conjugated detector pinholes.

4.3 Parameters that affect the lateral resolution of a CFM

4.3.1 General

Lateral resolution assessments are made by measuring the lateral size of a PSF on a CFM. The fundamental limit in the spatial resolution of a light microscope is given by:

Abbe Resolution$_{x,y}$ = λ/2NA, Abbe Resolution$_z$ = 2λ/NA^2

Comprehensive reviews of the relevant parameters and an experimental protocol for determining the size of a PSF are available elsewhere[2],[5],[6],[7].

4.3.2 Objective lens

The objective lens (OL) directly affects the lateral resolution of a CFM image because the size of the laser focus and the collection volume are determined by the NA of the OL.

4.3.3 Detection pinhole size and focal length of the tube lens

The confocal pinhole in front of a photo detector eliminates light originating outside of the focal volume in the sample. Smaller pinholes increase the resolution; however the amount of light that contributes to the image is also reduced, which can reduce the contrast in the image. Therefore the optimum size of the detection pinhole for a particular sample and application must be selected with the discretion of the operator. The imaged size of the detection pinhole on the sample plane by the tube lens and the OL is more important than the actual size of the detection pinhole. Therefore the magnification of the OL and the tube lens should also be specified.

4.3.4 Collimation and purity of the laser illumination beam

The expected performance of an OL can be achieved provided that the illumination beam is collimated and fills the back aperture of the OL.

4.3.5 Polarization of the laser illumination

The polarization of the input laser affects the shape and size of a PSF. Linearly polarized illumination tends to elongate the PSF along the direction of polarization[8].

4.3.6 Excitation and emission wavelengths

The wavelength of the light used in CFM has the direct effect on the size of PSF as described by Abbe resolution in 4.3.1. Furthermore, in the fluorescence imaging, the emission wavelength is different from (longer than) the excitation wavelength and this can lower the resolution[2].

4.3.7 Image contrast

Good contrast in a CFM image is required for the accurate measurement of the lateral resolution. The cleanness of the optics, signal strength, noise, sensitivity of the photo detector, throughput of the beam path, and beam polarization are empirical factors that affect the contrast of a CFM image.

5 Measuring the lateral resolution by imaging a small object

5.1 Background information

A very small object may be imaged to estimate the lateral resolution of a CFM. This approach is advantageous because one can obtain a two-dimensional profile that directly shows the PSF of the CFM from a single CFM image. The lateral resolution can be defined as the full width at half-maximum (FWHM) of the PSF. Because the finite size of a small object contributes to the observed size of the