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

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Contents

Page

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.

INTERNATIONAL STANDARD ISO 18337:2015(E)

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

object in the CFM image, the observed image of the object represents the convolution of the PSF and the spatial distribution of the small object. A wide range of samples may be used in this method, including fluorescent nanoparticles, such as light-emitting polymer nanoscale beads or QDs. Figure 1 shows the concept of the CFM measurement of fluorescent bead and the extraction of a line profile for the FWHM estimation.

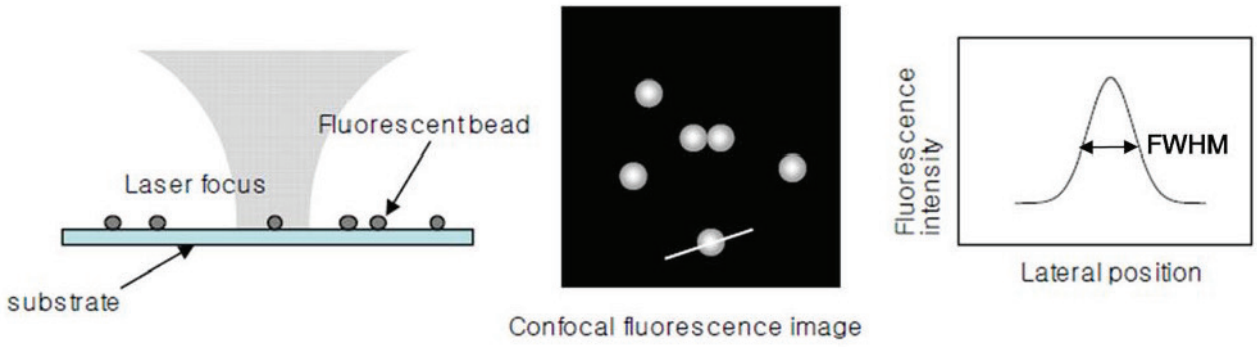


Figure 1 — A fluorescence image may be obtained, and the FWHM of the cross-sectional profile of a single bead is used to estimate the lateral resolution of a CFM instrument

5.2 Selection of the sample and sample requirements

If the size of the fluorescent nanoparticle is comparable to the size of the PSF in a CFM, a deconvolution process for deducing the actual PSF of the microscope is required. Deconvolution complicates the process of characterizing the PSF. The nanoparticles used here should be as small as possible; however, small particles produce a weaker fluorescence signal that can degrade the signal-to-noise ratio in a CFM image. Assuming that the PSF has a Gaussian profile, a particle less than one-fourth the size of the PSF will contribute less than 1 % to the resultant image[3] [4]. Considering the uncertainty of measuring the FWHM of a particle, this discrepancy may be taken as negligible. One-fourth the size of a typical PSF corresponds to 75 nm for a 300 nm PSF, indicating that one can use a 75 nm nanoparticle as a test specimen without worrying about deconvoluting the PSF from the obtained image. The apparent size of the nanoparticle in the image may then be taken as the lateral profile of the PSF. The shape of the fluorescent nanoparticle does not matter as long as the size in any direction of the nanoparticle is less than one fourth of the expected lateral resolution. Note that specified sizes of the manufactured fluorescent nanoparticles are not always correct with some variation depending on the manufacturer. The confirmation of the size of the nanoparticles can be obtained by using other high-resolution microscopes, such as atomic force microscopes and electron microscopes.

Nanoparticles must be immobilized on a clean substrate with low roughness, such as glass or a silicon wafer. If the nanoparticle is purchased in the form of a colloid, the colloid must be appropriately diluted prior to dispersal on the substrate. Colloidal solutions may be spread or spin-coated onto a substrate to an optimum density of several isolated particles per 100 μm^2. The cleanness of the substrate is very important. A substrate may be cleaned using a Piranha solution[9] or by ultrasonicating the substrate in an alkaline 1 M KOH solution, followed by washing with H_2O. Plasma cleaning is effective as well[10],[11].

5.3 Setting the parameters prior to operating the instrument

An image obtained by CFM depends on the operator's skill and the experimental parameters. Different CFM resolution values may be obtained for a given parameter set for different instrument operators. The extent to which a properly optimized experimental parameter set is achieved will depend on the operator. Therefore, this International Standard does not impose specific experimental parameters for use in operating a CFM instrument. Instead, the operator may choose the appropriate experimental parameters for optimized CFM imaging at his or her discretion. These parameters include but are not limited to the wavelength and beam quality of the excitation laser, the polarization of the illumination

beam, the alignment of the optics, and the spectral band of the detected fluorescent emission. Resolution and contrast are a different concept in theory but are closely interrelated in real imaging applications. Factors that can affect the contrast of an image are the laser intensity, S/N ratio, collection efficiency of the collection optics, and gains and sensitivity of the photo detector. Furthermore, the size of one pixel in an image should be less than one-fifth of the expected lateral resolution; that is, the range of the lateral resolution should be defined over at least five pixels[3] [4].

5.4 Data collection and analysis

5.4.1 Selecting a proper spot

CFM images are obtained by recording an image of the Stokes-shifted fluorescence. A CFM image desirable for the measurement of lateral resolution contains isolated bright spots, each of them corresponding to single fluorescent nano object, as shown in Figure 1. The experimental procedure of taking CFM image can vary depending on the instrument and the type of sample. A typical procedure is as follows.

1) Turn on the laser and make sure the optical path to the sample is clear with all optics in place.

2) Mount the highest available NA objective lens.

3) Place the prepared sample on the microscope stage and bring it to a tight focus by viewing into the eyepiece or camera monitor.

4) Turn or adjust necessary filter wheels or beam splitters so that the laser light reflected from the sample surface is detected by the photodetector.

5) With minimum laser power, while monitoring the photodetector output, perform the fine tuning of the focus by maximizing the photodetector output by adjusting the distance between the OL and the sample, or the position of detection pinhole position, if adjustable.

6) Use the smallest size of detection pinhole that still can provide a good contrast of the image. This step is largely dependent upon the operator's discretion.

7) After the fine focusing, insert the long pass filter or control the computer so that the long pass filter that blocks the laser light going into the photodetector is in place and increase the laser power.

8) If your instrument provides the real-time monitoring of photodetector output with line-scan and your sample is highly fluorescent, you can try to improve the focus and the alignment by monitoring the fluorescent light intensity and adjusting the focus and pinhole position with the long pass filter in.

9) Determine the scan direction, image size, the number of pixels, and the scan speed (or dwell time at pixels).

10) Perform the imaging and save the data for the analysis.

One isolated bright spot in the CFM image which represents a single nanoparticle is selected, and the FWHM is measured from a representative cross-sectional line profile of the single bright spot. The following conditions shall be met in selecting a bright spot.

1) The bright spot shall be isolated and not overlap with adjacent spots.

2) The observed bright spot shall be circular in shape. If the apparent aspect ratio of the longer axis and shorter axis exceeds 1.5, the estimation of lateral resolution is invalid, and an instrumental inspection is recommended.

5.4.2 Extracting a line profile using band-average process

The band-average process shall be used to obtain representative FWHM of a single spot profile. First select a rectangular region consisting of multiple (at least five are required) adjacent lines over a single fluorescent object spot. The average line profile is obtained from these lines that were used to form the

rectangular region. Figure 2 shows the process of band-average of line profiles over a single fluorescence spot. The band shall be chosen along both the short axis and the long axis of the bead profile. The resultant line profile, to be used for the estimation of FWHM, shall meet these two conditions.

1) The total length of the line profile should be at least five times the expected resolution[3].

2) The S/N ratio of a line profile used to measure the lateral resolution should exceed 5:1. The S/N ratio is the ratio of the central peak height to the baseline of the profile, and the maximum peak-to-peak value in the side plateau regions of the cross-sectional profile used to estimate the lateral resolution.

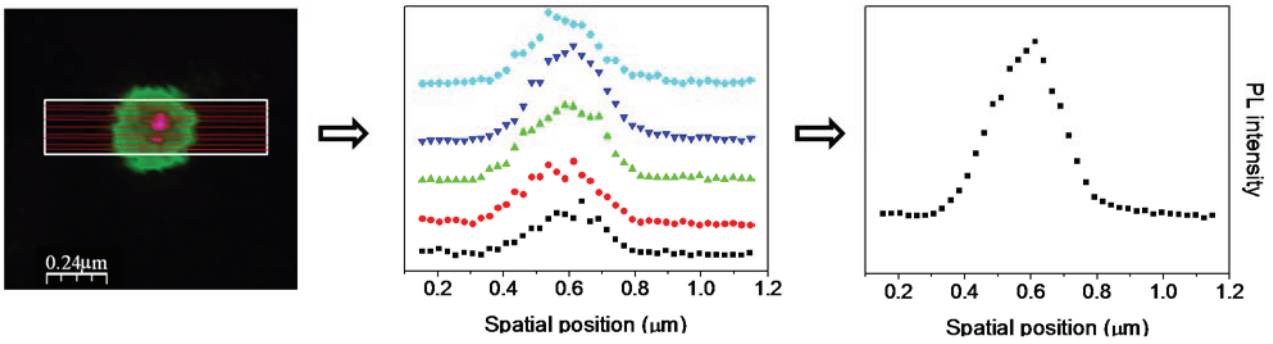


Figure 2 — Band-averaging of line-profiles to extract a representative line-profile to be used for the estimation of FWHM

5.5 Recording the data

The measured FWHM of the PSF is directly affected by the OL and the wavelengths of the laser excitation and detection. Therefore, the lateral resolution is meaningful only when these experimental parameters are properly described. Other relevant parameters that shall be provided are:

a) The type of CFM operation: Stage scanning or laser scanning.

b) The specification of the OL: air, oil-immersion, water-immersion, NA, and magnification.

c) The size of the detection pinhole in units of Airy disk, or the tube lens magnification and the actual size of the pinhole. The above mentioned projected pinhole size is an alternative parameter that can be stated here.

d) Excitation wavelength and detection wavelength.

e) Polarization state of the input light.

f) Power of the laser focus on the sample.

g) Sample description: The known size of the nanoparticle or QD used for imaging.

h) The size of the image field in μm.

i) Number of pixels in the x and y directions.

j) The fast scanning direction.

k) The S/N ratio of the line profile selected for measuring the lateral resolution.

l) The FWHMs of a single particle in a CFM image in the x- and y-axes.

Annex A
(informative)

Sample preparation and example of data and analysis

A.1 Sample preparation

An example of the measurement of the lateral resolution of CFM using fluorescent nanobeads as a test sample is presented.

Any type of the nanoparticle immobilized on a flat substrate may be used to determine the PSF provided the particle can be approximated as a point light source. The nanoparticle lateral dimension should not exceed one-fourth of the expected resolution.

The nanoparticles must be immobilized on a level substrate, such as glass or a silicon wafer. If the nanoparticle is purchased in the form of a colloid, the colloid must be appropriately diluted prior to dispersal on the substrate. Colloidal solutions may be spread or spin-coated onto a substrate to an optimum density of several isolated particles per 100 μm^2. The cleanness of the substrate is very important. A substrate may be cleaned using a Piranha solution[6] or by ultrasonicating the substrate in an alkaline 1 M KOH solution, followed by washing with H_2O. Plasma cleaning is effective as well [7],[8].

The actual procedure used to prepare the sample is as follows:

1) A cover glass (Menzel–Glasser) was cleaned sequentially by dipping in KOH (1 M) then washing with acetone and water in an ultrasonic bath.

2) The KOH washing step proceeds for a longer period of time (30 min) than the acetone or water washing steps (15 min each). The KOH dissolves inorganic materials attached to the cover glass, whereas the acetone removes organic materials. Finally, water was used to wash everything from the cover glass.

3) Process 2) was repeated two or three times, and the substrates were dried by gently blowing air across the surfaces.

4) A fluorescent bead suspension with an average size of 20 nm was purchased from Invitrogen Corp. and diluted in water to prepare solutions with concentrations of 1/50,000, 1/100,000, and 1/200,000.

5) A 5 µL volume was dispersed on a clean cover glass by spin-coating at 5000 rpm for 60 sec.

In this case, a 1/200,000 diluted sample yielded a density of tens of particles per 100 μm^2 area.

A.2 Data acquisition and analysis

The CFM of an Alpha300 system manufactured by the Witec Company in Germany was used here, and the lateral resolution was measured by fluorescent imaging of the sample described in 5.1. The experimental procedure used to obtain the images of the particle are described in detail below:

1) The sample was placed on the microscope stage and the sample region was confirmed by focusing white light through the 100x, 1,49 NA oil-immersion objective of the CFM.

2) A laser beam with a wavelength of 488 nm at a low power on the order of microwatts was focused onto the sample, and the PMT output was optimized.

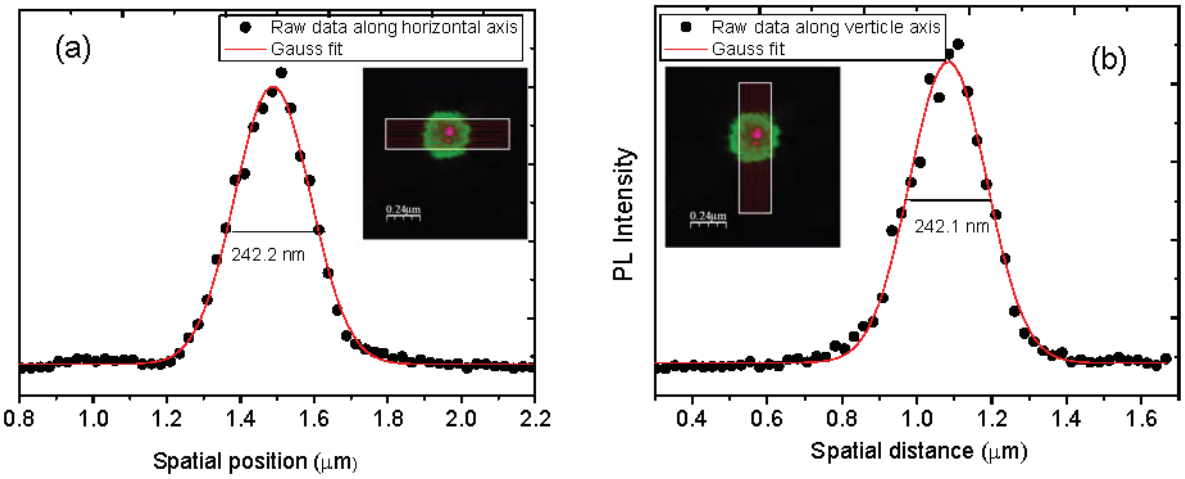
3) During optimization of the optical signal, the reflected laser light was collected through the objective used for illumination and was focused through a pinhole in front of the detector. Special care was

given to optimize the PMT alignment by repeating the process of optimization. This ensured that only light from the image focal plane could reach the detector, thereby significantly increasing the image contrast.

4) In principle, smaller detection pinholes yield higher lateral resolutions; however, a smaller pinhole reduces the amount of detected light and can reduce the image contrast. A 50 µm core multimode fibre was used to collect the fluorescence light in our measurement. This core size corresponded to about twice the size of an Airy disk.

5) The laser was raster-scanned across the sample by scanning the sample stage, and the CFM image was acquired.

Figure A.1 shows two representative line profiles of the same single bead fluorescence image obtained by band averaging across the x and y directions. Average line profiles were fit to Gaussian profiles. The $FWHM_x$ and $FWHM_y$ values were measured to be 242,2 ± 3,8 nm and 242,1 ± 3,8 nm, respectively.



Key

(a) x-axis band average

(b) y-axis band average

Figure A.1 — Band averaged line profile of the same spot

A.3 Recording the data

Type of CFM operation	Stage scanning type
Specification of the objective	Oil immersion objective. NA 1,49, 100x Zeiss
Detection pinhole size	~2 Airy disks (50 µm with 1x tube lens)
Laser wavelength	488 nm
Input polarization	Unidentified
Laser power	2–3 µW at the sample
Sample description	5 µL yellow fluorescent bead (Invitrogen Corp.) aqueous solution spin-coated onto a cover glass.
The size of the nanobeads	approximately 20 nm diameter
Image size	1,5 × 1,5 μm^2
Pixel number	60 × 60 pixels
Fast scan direction	x-axis
Speed of line scanning	0,33 Hz
FWHMx (nm)	242,2 ± 3,8
FWHMy (nm)	242,1 ± 3,8

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