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A new method for depth profiling reconstruction in confocal microscopy

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A B S T R A C T

Confocal microscopy is commonly used to reconstruct depth profiles of chemical species in multicomponent systems and to image nuclear and cellular details in human tissues via image intensity measurements of optical sections. However, the performance of this technique is reduced by inherent effects related to wave diffraction phenomena, refractive index mismatch and finite beam spot size. All these effects distort the optical wave and cause an image to be captured of a small volume around the desired illuminated focal point within the specimen rather than an image of the focal point itself. The size of this small volume increases with depth, thus causing a further loss of resolution and distortion of the profile. Recently, we proposed a theoretical model that accounts for the above wave distortion and allows for a correct reconstruction of the depth profiles for homogeneous samples. In this paper, this theoretical approach has been adapted for describing the profiles measured from non-homogeneous distributions of emitters inside the investigated samples. The intensity image is built by summing the intensities collected from each of the emitters planes belonging to the illuminated volume, weighed by the emitters concentration. The true distribution of the emitters concentration is recovered by a new approach that implements this theoretical model in a numerical algorithm based on the Maximum Entropy Method. Comparisons with experimental data and numerical simulations show that this new approach is able to recover the real unknown concentration distribution from experimental profiles with an accuracy better than 3%.

1. Introduction

The optics geometry in confocal microscopy is designed to significantly increase the contrast, and hence the visibility of fine details in the specimen [1,2], by excluding most of the out-of-focus light from the final image due to the presence of the pinhole on the image screen. This technique is particularly suited to perform optical depth profiling and optical sectioning of thick specimens. Several contributions are available in the literature where depth scanning mode in confocal microscopy has been exploited to investigate both biological and inorganic specimens, in combination with different optical techniques, such as reflectance, fluorescence, Raman scattering [3–8].

It is worth noting, however, that a true confocal image of a plane is obtained only in the ideal case of a laser beam with diameter approaching to zero. In fact, in real cases, the confocal microscopy does not image the light emitted from a plane at depth z but rather that emitted from a small volume around z, due to several physical phenomena. The most relevant are diffraction phenomena of the optical system, the mismatch of the refractive index between the specimen and the medium surrounding the objective lens and the finite size of beam spot. Many theoretical approaches have been proposed to address these issues, accounting for the wave distortion of the confocal measurements with the principles of optics [6,9–15]. Recently, our group has proposed a model for the description of the actual spatial distribution of the measured intensity in a confocal microscopy depth profiling measurement [16] that considers the effects of the refractive index mismatch by treating the surface of separation between the sample and the medium surrounding the microscope objective as a diffraction plane. To this aim, the second Rayleigh-Sommerfeld diffraction integral [17], within the framework of the scalar wave theory, has been used to describe the diffraction effects and the propagation of the distorted field through a system of two converging lenses. This model is suitable to describe the light emitted from homogeneous samples and detected with a confocal configuration for any excitation source and it has been successfully validated for the particular case of Raman scattering emission from different materials. Starting from these previous results, in this contribution we tackle the complex 'inverse' problem of performing the correct reconstruction of unknown profiles of chemical species and moieties within a matrix based on depth profiling experimental information gathered by confocal microscopy. To this purpose, the total intensity detected through the pinhole, that is an image point, is written as the sum of the intensity from the emitters planes at different depths weighted by the emitters concentration in the framework of the theoretical model proposed in Ref. [16]. A new procedure based on the Maximum Entropy Method (MEM) has been developed for recovering the true distribution of the excited emitters. The MEM is generally applied as a tool for image analysis and image improvement in several disciplines such as radio astronomy, medical imaging, pulse fluorimetry, fluorescence spectroscopy, etc. The deconvolution of high resolution transmission electron microscope images, the decrease of the reconstruction artifacts that affect the scanning transmission electron microscopy and the increase of the image resolution in scanning tunneling microscopy are only a few examples of the successful implementation of the MEM procedure in microscopy [18–20]. From

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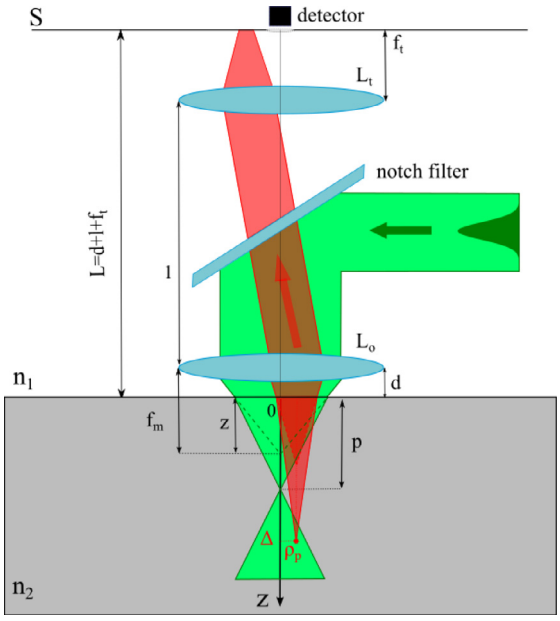

Fig. 1. Schematic representation of a confocal Raman microscope. The green strip is the input source that is focused by the objective lens L_0 with focal distance f_m. Σ is the interface between the sample (with a refractive index n_2) and the surrounding medium (with a refractive index n_1). The nominal focal position of the beam is $z = f_m - d$ and the actual one is $p = (n_2/n_1)\,z$. A point-like emitter located at a depth Δ and radial distance ρ_p emits radiation (red strip) that propagates through the two converging lenses up to the image screen S. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

the mathematical point of view, the MEM is a powerful algorithm that does not rely upon predetermined functional form, avoiding the introduction of fitting parameters with a limited or no physical meaning. The task is accomplished by using a "regularizing function", as the well-known Shannon-Jaynes entropy, which is maximized subject to the goodness of fit parameter constraint $\chi^2 \cong 1$ [21–23].

The accuracy and the efficacy of the proposed procedure is tested through comparison with both experimental data and 'virtual' data obtained by numerical simulations. First, the MEM algorithm is applied to the analysis of the experimental depth profile of Raman intensities collected in the case of a homogeneous specimen, a polystyrene sheet. Although the data refer to the simple case of a uniform profile, the experimentally determined depth profiling is characterized by the mentioned pitfalls and artifacts, if not properly processed by MEM algorithm. Then, the proposed approach is used to elaborate data obtained in the case of a non-uniform distribution of emitters in a multi-component system. Due to lack of experimental data, a simulated Raman depth profile has been generated, as it would experimentally result in the case of diffusion of a low molecular weight compound through a plane polymeric sheet.

The main objective fulfilled by the present investigation has been the development of a procedure allowing to tackle the general problem of determining the concentration profile of different species in multicomponent systems. Relevant practical examples where such procedure for reconstruction of concentration profiles can be effectively used are controlled drug delivery, determination of distribution of species in living tissues, membranes for separation of mixtures of gases and vapors, evaluation of the effects of environmental humidity on durability of polymeric materials and migration of additives in polymeric systems [24–27].

2. Theory

The typical configuration of a confocal microscope is reported in Fig. 1. In detail, a Gaussian laser beam (green strip), with excitation wavelength λ_{ex}, is transmitted through the notch filter and focused below the interface Σ by the objective lens L_0 whose focal distance is f_m. Due to the mismatch of the refractive index, at Σ, between the medium (with refractive index n_1) which surrounds the lens L_0 and the sample (with refractive index n_2), the beam focus shifts from the position $z = f_m - d$ below Σ to $p = (n_2/n_1)z$ [28].

The spatial distribution of the intensity of the radiation emitted from the confined excitation volume is given by:

$$I_{ill}(\rho_p, \Delta, z) = I_0 \left[\frac{w_0}{w(\Delta, z)}\right]^2 e^{-\frac{2\rho_p^2}{w^2(\Delta,z)}}, \tag{1.1}$$

where ρ_p *and* Δ are, respectively, the radial distance from the optical axis and the depth of an illuminated point. The quantity *w(Δ,z)* is the beam-waist radius and is given by the following formula:

$$w(\Delta, z) = w_0 \sqrt{1 + \left(\frac{\Delta - (n_2/n_1)z}{n_2 z_0}\right)^2}, \tag{1.2}$$

where $z_0 = \pi w_0^2/\lambda_{ex}$ is the Rayleigh range and w_0 is the minimum value of w(Δ, z) that is observed at the actual focus position $p = (n_2/n_1)z$ with the maximum intensity I_0.

An illuminated unit point source at (ρ_p, θ_p, Δ) emits light with wavelength λ_{em} that propagates through the spatial configuration of the confocal microscope (red strip in Fig. 1) back to the tube lens L_t with focal distance f_t where is focused on the image plane S. The propagated field has been extensively discussed in Ref. [16] and its value in a point at radial distance r' on the image plane S is described by the following expressions:

$$\begin{aligned} U(r', L|\Delta) &\propto \frac{\Delta}{M}\int_0^\infty \frac{e^{(-\imath n_2 k_{em} s)}}{s}\left(\frac{\imath n_2 k_{em}}{s} + \frac{1}{s^2}\right) \times e^{\imath f r^2} J_0(wr) r\,dr \\ k_{em} &= \frac{2\pi}{\lambda_{em}} \quad s = \sqrt{\Delta r'^2 + \Delta^2} \quad f = \frac{n_1 k_{em}}{2z} \quad w = \frac{n_1 k_{em} \Delta r'}{M z} \\ M &= \frac{f_t}{f_m} \quad \Delta r' = \sqrt{r'^2 + M^2\rho_p^2 - 2Mr'\rho_p \cos(\theta' - \theta_p)} \end{aligned} \tag{1.3}$$

Consequently, the spatial distribution of the intensity of the image source illuminated by the focused beam laser is given by:

$$I_p(r', L|\Delta) = I_{ill}(\rho_p, \Delta, z)\frac{|U(r', L|\Delta)|^2}{2\pi\int_0^\infty r'|U(r\prime, L|\Delta)|^2 dr'} \tag{1.4}$$

The power that flows through the pinhole of radius *R* centered on the optical axis does not depend on the angular position θ_p of the emitter and can be expressed as:

$$P_p(\rho_p, z, \Delta) = 2\pi\int_0^R \int_0^{2\pi} r' I_p(r', \theta'|\rho_p, \theta_p, z, \Delta)\,dr'\,d\theta'. \tag{1.5}$$

The signal resulting from the emission by a set of random emitters on the plane at depth Δ:

$$P(z, \Delta) = 2\pi\int_0^\infty r_p P_p(\rho_p, z, \Delta)\,dr_p. \tag{1.6}$$

while the total intensity detected through the pinhole can be calculated as:

$$P_{tot}(z) = \int_0^\infty c(\Delta)P(z, \Delta)d\Delta. \tag{1.7}$$

where *c(Δ)* is the concentration of the emitters along the optical axis. The quantities *c(z)* and $P_{tot}(z)$ are, respectively, the true and apparent profiles.

The Eq. (1.7) can be discretized as follows:

$$P_{tot}(z_i) = \sum_{j=1}^{N} c_j P_{i,j} c_j \equiv c(\Delta_j)P_{i,j} \equiv P(z_i, \Delta_j) \tag{1.8}$$

For a focusing nominal depth z_i, the intensity $P_{i,j}$ of the plane emitters at depth Δ_j is weighted by the emitter concentration c_j.