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Nondestructive evaluation of polymeric paints and coatings using two-photon laser scanning confocal microscopy

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A 120 μm thick tri-layer polymer paint on an aluminium substrate was imaged in three-dimensions using multichannel two-photon confocal laser scanning microscopy. Each layer is doped with a different fluorophore so that the fluorescence emission from the layers can be separated into different channels. Use of i.r. excitation light allows deeper penetration in the polymer layers. The image reveals fine details within each layer, as well as information about the interfaces between layers. The substrate features are also visible with excellent resolution in negative contrast. This technique provides a nondestructive method to probe thick multilayer polymer materials. © 1997 Elsevier Science Ltd.

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Introduction

Confocal microscopy has become a commonly used tool to obtain three-dimensional (3D) images of biological specimens such as tissues and cells[1–3]. Although confocal microscopes can be used in different imaging modes, the most widely used mode of operation is fluorescence imaging. Other modes of operation, such as reflective imaging are generally used in material, dental and ophthalmic imaging[4]. A confocal microscope in fluorescence mode detects the fluorescence emission from a sample, which is either intrinsically fluorescent or is stained with a fluorescent probe. Its ability to generate 3D data arises from the fact that a confocal microscope has excellent depth resolution unlike other bright field optical microscopes. To achieve the axial resolution, a confocal pinhole is introduced in the optical system which allows only signals at the focal point to pass through and reach a photodetector. In this way, out-of-focus signals are eliminated. *Figure 1* shows the schematic of a typical confocal laser scanning microscope (CLSM). Note that the rays representing fluorescence originating above and below the focal plane are blocked by the confocal aperture. By changing the focal plane, it is possible to optically section the sample to depths of over 100 μm.

Fluorescence confocal microscopy is usually performed by single-photon excitation of a fluorophore by u.v. or visible light, preferably a laser source for convenience. The emitted fluorescence, which is at a longer wavelength than the excitation source, is detected by a photodetector, commonly a photomultiplier tube (PMT) or CCD, depending on the type of instrument used. In a CLSM, a scanning mechanism raster scans the beam over a plane of the volume to be imaged. By repeating this process over successive planes along the optical axis, a complete 3D image of the sample can be obtained. Fluorescence confocal microscopy is already widely used to image biological specimens where a fluorescent molecule acts as a probe. By tagging specific antibodies, the fluorophore

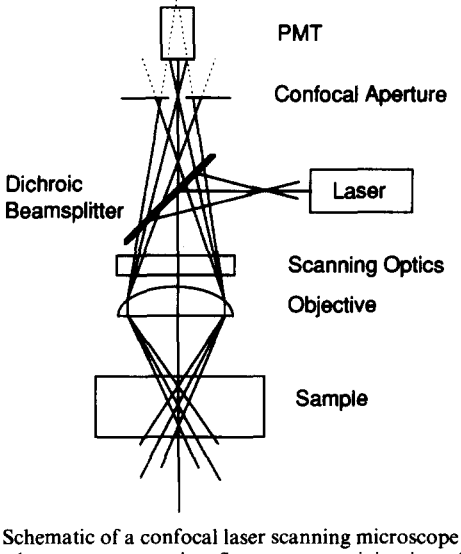

Figure 1 Schematic of a confocal laser scanning microscope (CLSM). Note that the rays representing fluorescence originating above and below the focal plane are blocked by the confocal aperture. By changing the focal plane, it is possible to optically section the sample. Two-photon excitation offers intrinsic optical sectioning, thus eliminating the need for a confocal aperture

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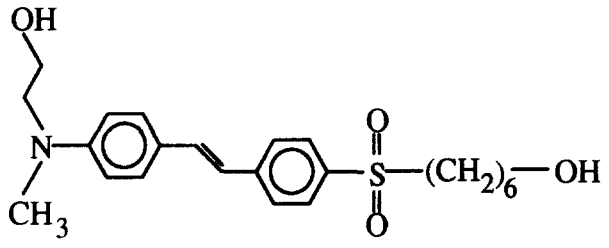
(a)



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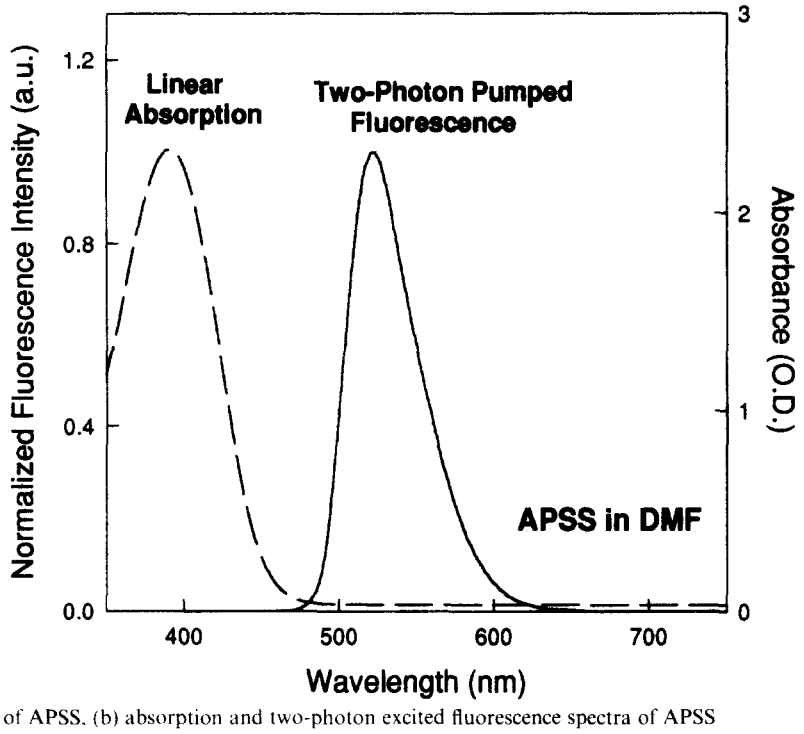

Figure 2 (a) Molecular structure of APSS, (b) absorption and two-photon excited fluorescence spectra of APSS

can be made to selectively localize at specific structures within a cell. Also, by selecting fluorophores which emit in different spectral regions, along with different antibodies, it is possible to probe multiple structures within a cell (multichannel microscopy), and study their localization and spatial relationship.

The use of two-photon excitation in fluorescence microscopy was introduced[5] by Denk *et al.* in 1990 and demonstrated by others both in biological and material samples[6,7]. In two-photon confocal microscopy, one uses direct two-photon pumped nonlinear absorption to excite a fluorophore which then fluoresces. In this process, the pump wavelength is longer (towards the i.r.), while the emission is upconverted to the visible. Two-photon confocal laser scanning microscopy (2PCLSM) has several advantages over its single-photon counterpart. One of the obvious advantages arises from the quadratic dependence of the two-photon induced fluorescence intensity on the excitation intensity. Under proper excitation power, this property limits the fluorescence emission to the vicinity of the focal point, thus providing excellent depth resolution even without using a confocal aperture. The excitation wavelength in 2PCLSM is in the near infrared as opposed to u.v. or visible for its single-photon (1PCLSM) counterpart. The penetration depth of the u.v. or even visible excitation light can be poor in many organic materials where the linear attenuation and scattering are high. This normally limits the depth in a material that one can probe using 1PCLSM. Near i.r. light, on the other hand, has a much greater penetration in polymeric materials. Furthermore, although fluorescence data are obtained from the vicinity of the focal point in a single-photon fluorescence confocal microscope, the entire specimen volume is illuminated and fluoresces, leading to photobleaching of the fluorophore in the entire illuminated volume.

We recently demonstrated the use of 2PCLSM as a nondestructive evaluation technique for polymers[8]. This technique provides a significant advantage in the gain of three-dimensional resolution and depth of probing. In

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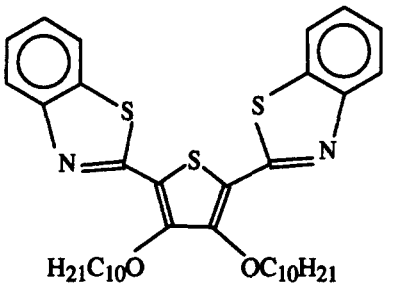

(b)

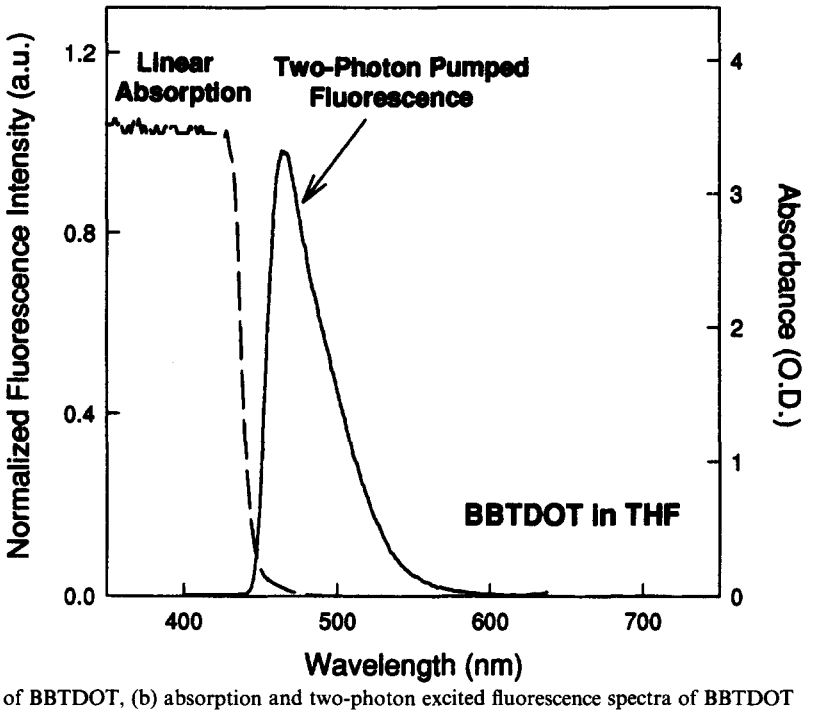

Figure 3 (a) Molecular structure of BBTDOT, (b) absorption and two-photon excited fluorescence spectra of BBTDOT

that report, we presented images of fluorophore-doped polymer blocks to a depth of 200 μm with submicron resolution. Due to the large penetration depth of the excitation light, one can easily investigate the surface, the bulk, and any underlying fractures or defects. The strong signal obtained in those images was due to the use of a new fluorophore designed and synthesized in our laboratories for this application. For 2PCLSM of polymers, the fluorophore may be an integral part of the molecular structure of the polymer system being investigated or just physically dispersed (solid solution).

In this communication, we demonstrate multichannel 2PCLSM as a tool to nondestructively probe the surface and interfaces in multilayered paint coatings. By virtue of the longer penetration depth of near infrared light in many materials, it is possible to image through several layers of paint coatings down to the substrate. Another advantage of using i.r. excitation is that u.v. excitation normally used in 1PCLSM could lead to chemical damage of the coating or paint, whereas the i.r. excitation is relatively safe. By using different fluorophores in different layers of coatings, one can conduct multichannel 2PCLSM to selectively probe each layer.

Experimental

The sample that was used to demonstrate multichannel 2PCLSM of polymer coatings consisted of three layers of epoxy paint (Hobbypoxy epoxy enamel, Pettit Paint Co., NJ) on an aluminium substrate. The top and bottom layers were doped with two new fluorophores, 2,5-bisbenzothiazole 3,4-didecyloxy thiophene (BBTDOT) and 4-[N-(2-hydroxyethyl)-N-(methyl) amino phenyl]-4'-(6-hydroxyhexyl sulfonyl)stilbene (APSS), both of which absorb 800 nm light by two-photon absorption, but fluoresce at different wavelengths (460 nm and 520 nm respectively). Thus, the top layer emits blue fluorescence while the bottom layer fluoresces green, enabling two-channel detection. The intermediate layer was undoped.

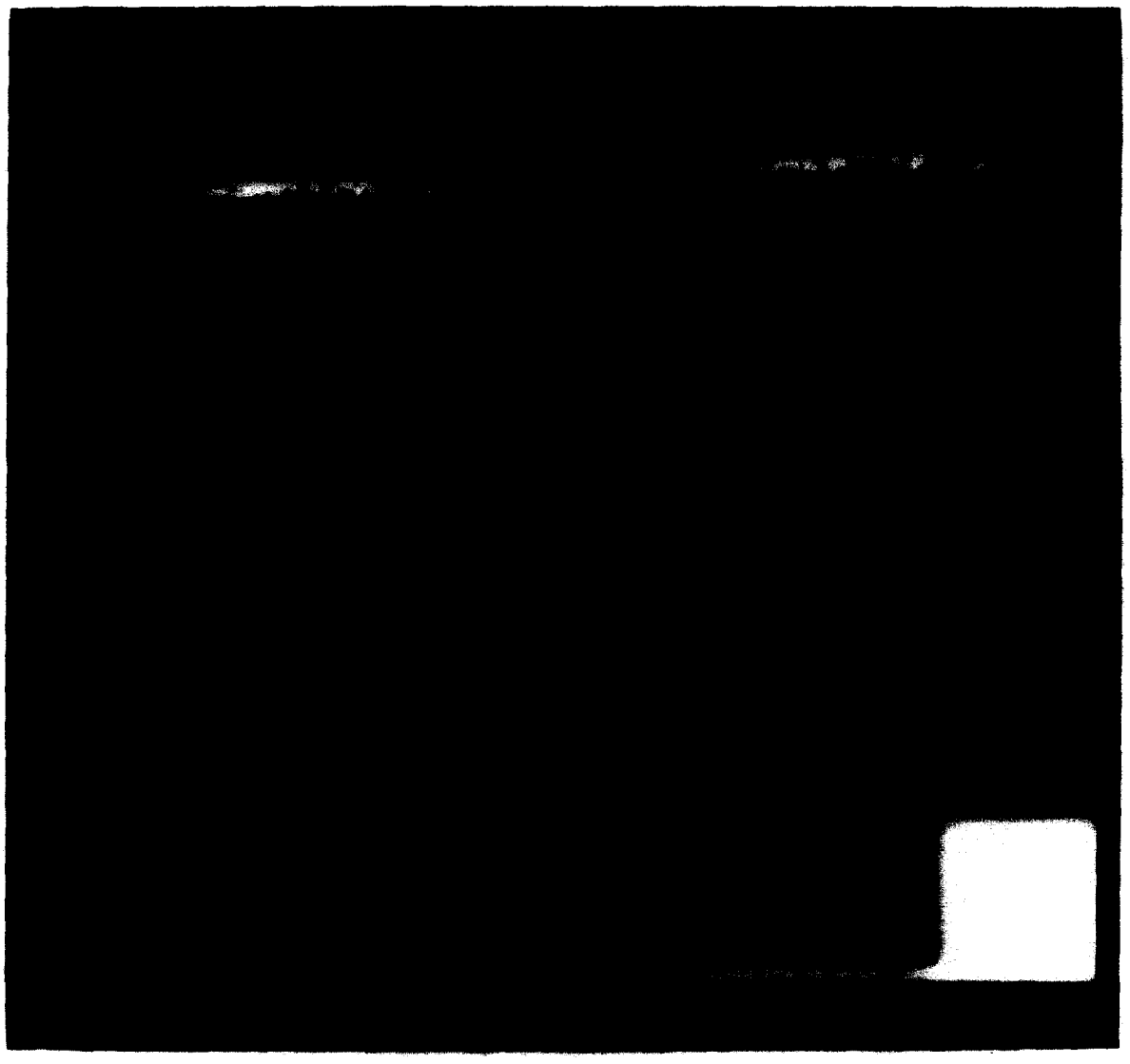

Figure 4 (a), (b), and (c) Different cut planes of a reconstructed three-dimensional data set. (d) Views of the same reconstructed data set in x–y, x–z and y–z planes. Total thickness of the paint layers is about 120 μm

Figure 2 shows the molecular structure, the linear absorption spectrum and two-photon pumped fluorescence spectrum of APSS[8], while *Figure 3* shows the same for BBTDOT[9]. The fluorophores were dissolved in the paint at a concentration of about 1% wt/wt. The fluorophore-doped paints exhibited fluorescence spectra characteristic of the corresponding fluorophores under both single and two-photon excitation. The fluorophore APSS has also been shown to perform as an efficient two-photon pumped lasing medium[9].

The images presented here were obtained with excitation from a mode-locked Ti : sapphire laser oscillator (NJA-4 from Clark-MXR Inc.) producing a train of 798 nm pulses of duration 90 fs each, at a frequency of 92 MHz. The average power in the beam was 400 mW. With the use of a beamsplitter and beam steering optics, about 30% of the beam was diverted into the entrance port of a modified BioRad MRC 500 laser scanning confocal microscope. The losses due to the various optics in the excitation path, and overfilling of the back aperture of the objective and the entrance pupil, reduce the actual average power at the location of the sample to only 0.3 mW. Still, due to the high two-photon pumped fluorescence efficiency of the fluorophores, 0.3 mW of average power from 90 fs pulses (low average power but high peak power) was sufficient to generate significant fluorescence which enabled the use of type II detection system (detection through a confocal aperture). The objective lens used for all images presented here, was either a Nikon Fluor-40X (NA = 1.3) or an Olympus S-PlanApo 60X (NA = 1.4). A 800 nm reflecting dichroic beam splitter and a 520 nm short pass filter were used in the detecting path of the confocal system. A second dichroic beam splitter further separated the emission from the two fluorophores and directed the beams to two photomultiplier tubes.

Results and discussion

Using the setup described above, it was possible to image through the entire thickness of all three layers (120 μm). *Figures 4a–c* show different cut planes of a reconstructed three-dimensional data set. *Figure 4d* shows views of the same reconstructed data set in x–y, x–z and y–z planes. The top and bottom layers have been assigned false colours by a computer, based on the signal strength between the two detecting channels, for better visual discrimination. Such images can yield information about the layer thickness, bonding between layers,

Figure 5 Different optical sections (at 0, 2, 4, and 6 μm in depth measured from the substrate surface) of the aluminium substrate imaged in negative contrast mode

and diffusion across layers (note the gradual changes in fluorescence intensity between the adjacent layers).

In addition, optical sectioning down to the substrate can yield the surface profile of the substrate. The substrate had been sanded with a coarse sandpaper to create a rough surface before being coated with paint. *Figure 5* shows surface-shaded profiles of different optical sections of the aluminium substrate by negative contrast imaging mode.[7,10]. In positive contrast imaging, the objects to be imaged (the paint layers in this case) are fluorescent while the remaining regions are dark. As opposed to this, negative contrast imaging implies that the objects to be imaged (aluminium substrate in our case) are dark while the surrounding regions (paint, which follows the substrate surface profile) are fluorescent. Note the fine surface features on the substrate. The four images show various optical sections starting with the surface of the substrate (top left image), to a depth of 6 μm in the ridges on the substrate (bottom right). As expected, the fluorescence intensity drops as one probes deeper, since the aluminium substrate does not fluoresce. When the focal point reaches the bottom of the deepest ridge on the substrate, the signal level drops to zero.

Although the images presented here were polymeric paints, this technique can be generalized to any type of multi-layer coatings as long as they are relatively transparent to the excitation wavelength (near i.r.) and to the fluorescence wavelength. Two-photon confocal laser scanning microscopy is, therefore, not limited to biological samples, but can also be used effectively in material science applications such as nondestructive evaluation of polymers and other insulating media, and for probing multilayer paints and coatings.

Acknowledgements

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