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Colour confocal reflection microscopy using red, green and blue lasers

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SUMMARY

To obtain colour reflected confocal images we have incorporated three lasers (HeNe: 633 nm; NdYAG: 532 nm; HeCd: 442 nm) and three photomultiplier detectors into our on-axis scanning system then adjusted the registration of the simultaneous output signals to produce full-colour images on a video monitor. Colour confocal images were produced from multi-stained fixed tissue as well as from natural pigments in fresh plant material. Rayleigh scattering properties of immunogold-labelled specimens were studied to show how variations in colour response can be utilized to identify subwavelength gold particles. Colour stereo pairs were produced to illustrate the accuracy with which the three-laser microscope system can record depth information without incurring problems due to chromatic aberration effects.

INTRODUCTION

The confocal scanning optical microscope is well known to have useful imaging properties not possessed by conventional microscopes: it has somewhat improved lateral resolution and, perhaps most importantly, it has the property of optical sectioning (improved axial resolution). For viewing thick objects, this latter feature allows the microscopist to produce a series of images from consecutive planes of focus which can then be combined digitally to form three-dimensional reconstructions (Carlsson *et al.*, 1985).

In the biological sciences, the term confocal microscopy is often assumed to be strictly synonymous with confocal fluorescence microscopy. There exists, however, a wide range of alternative confocal techniques such as reflected bright-field (Cogswell & Sheppard, 1990a), differential phase contrast (Benschop, 1987) and differential interference contrast (Cogswell & Sheppard, 1990b) which can retrieve amplitude and/or phase information and are based on the analogous methods of conventional microscopy. In general, these techniques, properly utilized, offer the usual advantages of confocal microscopy for observing the absorbing and reflecting properties of samples at some pre-determined, usually monochromatic, illuminating wavelength. A problem

arises in confocal microscopy, however, when attempts are made to image accurately the overall spectral properties of specimens.

The ability of the human visual system to detect colour is, and has been for decades, one of the primary factors underlying the design of conventional microscopes and the preparation of specimens (be they utilized in biological, geological or materials science) which may span applications as diverse as the location of particular biological tissues by incorporation of vital stains or techniques to determine crystalline mineral phases in polarizing microscopy. To date, however, there are only a few commercially available confocal instrument designs that have the ability to produce full-colour images and these certainly have limitations. For example, confocal microscopes which use white-light sources and employ rotating Nipkow discs are easy to operate and can produce useful full-colour images; however, these images frequently display chromatic aberration effects which can create an undesirable loss in resolution. On the other hand, these chromatic effects may sometimes be put to good use to provide additional information. For example, Boyde (1987) has used the natural chromatic aberration of a microscope objective to colour-code images in the confocal tandem-scanning microscope. Similarly, Molesini *et al.* (1984) have introduced chromatic aberration in a laser system in order to form profiles of surface topography. However, in the tandem-scanning designs, because the different wavelengths of light pass through the same pinholes, it is not possible to adjust independently the foci of the various colours in order to cancel out or selectively introduce chromatic aberration at will. (This will be shown to be one of the advantages of our three-colour confocal microscope described below.)

Another design of confocal microscope, which utilizes three laser light sources in a beam-scanning configuration, is produced by at least one commercial manufacturer (Lasertec model 2LM11; Awamura *et al.*, 1987). However, due to the inherent properties of beam-scanning designs (Cogswell & Sheppard, 1990a), it is extremely difficult to control the chromatic path differences which occur as the beam scans across the field. In addition, determining the optimum axial position for each of the three (red, green and blue) detector apertures is further complicated by the fact that different objectives must be utilized when a magnification change is desired.

In view of these limitations, in this study we have chosen to utilize an on-axis specimen-scanning confocal microscope configuration with three laser illuminators and three detectors. This microscope design allows us to eliminate off-axis chromatic effects and to fine-tune the system to one single high-NA objective through the addition of small-correction opticians' lenses (Cogswell *et al.*, 1990). If a change in magnification is then required, we can continue using the same objective and simply alter the distance over which the object is scanned.

THE CONFOCAL SCANNING OPTICAL MICROSCOPE

Figure 1(a) illustrates the basic principles of an on-axis scanning optical microscope operating in reflection. The laser beam is expanded to illuminate the objective lens, which focuses a diffraction-limited spot onto the object plane. The object is scanned across the spot in a pattern in the x–y plane similar to a video raster. Light reflected from the specimen passes back through the objective lens, is reflected from the half-silvered mirror and falls onto the photodetector, whose output, after amplification, modulates the brightness of a video display (scanned simultaneously with the object) to form an image.

Depending upon whether the detector is of large area or a point, there are two forms of the scanning optical microscope, often referred to as Type 1 (conventional) and Type 2 (confocal) (Sheppard & Choudhury, 1977). In the Type 1 microscope, the imaging performance is determined by just the diffraction-limited spot of light projected onto the specimen by the objective lens, with the detector responding to a

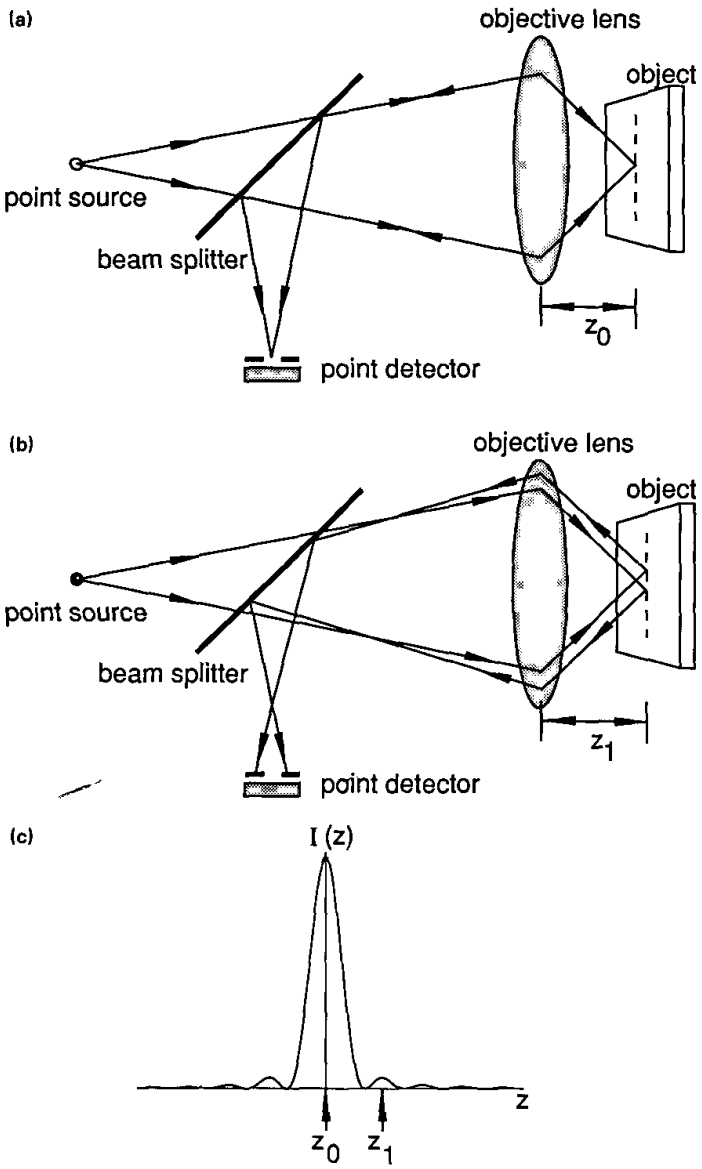

Fig. 1. (a) (b) Schematic diagrams of the reflection confocal scanning optical microscope, showing the change in position of the focused image with respect to the detector pin-hole as the object (mirror) is moved out of the focal plane. (c) A typical defocus response curve with intensity $I(z)$ plotted along the vertical axis and the focus position of the objective (z position) along the horizontal axis, with z_0 and z_1 corresponding to the focus positions in (a) and (b).

the light reflected from the object. In this configuration, as far as the reflected light component is concerned, the objective lens acts merely as a collector. The imaging performance of this form of scanning system is identical with that of a conventional microscope. In the confocal configuration, which utilizes a pin-hole in front of the detector, the object is still illuminated with a diffraction-limited spot, but reflected light is collected only over the area of the pin-hole. The point detector can be thought of as defining a region on the object which is, since the same objective is used for both the incident and reflected beams, another similar diffraction-limited spot. The effective point-spread function (PSF) of this arrangement is thus narrower than in the conventional microscope.

In addition to an improved PSF, the confocal microscope has the property of optical sectioning. If the object is moved out of the focal plane as shown in Fig. 1(b), reflected light is no longer brought to a focus on the point detector, with the result that the strength of the detected signal drops significantly. Out-of-focus information from the

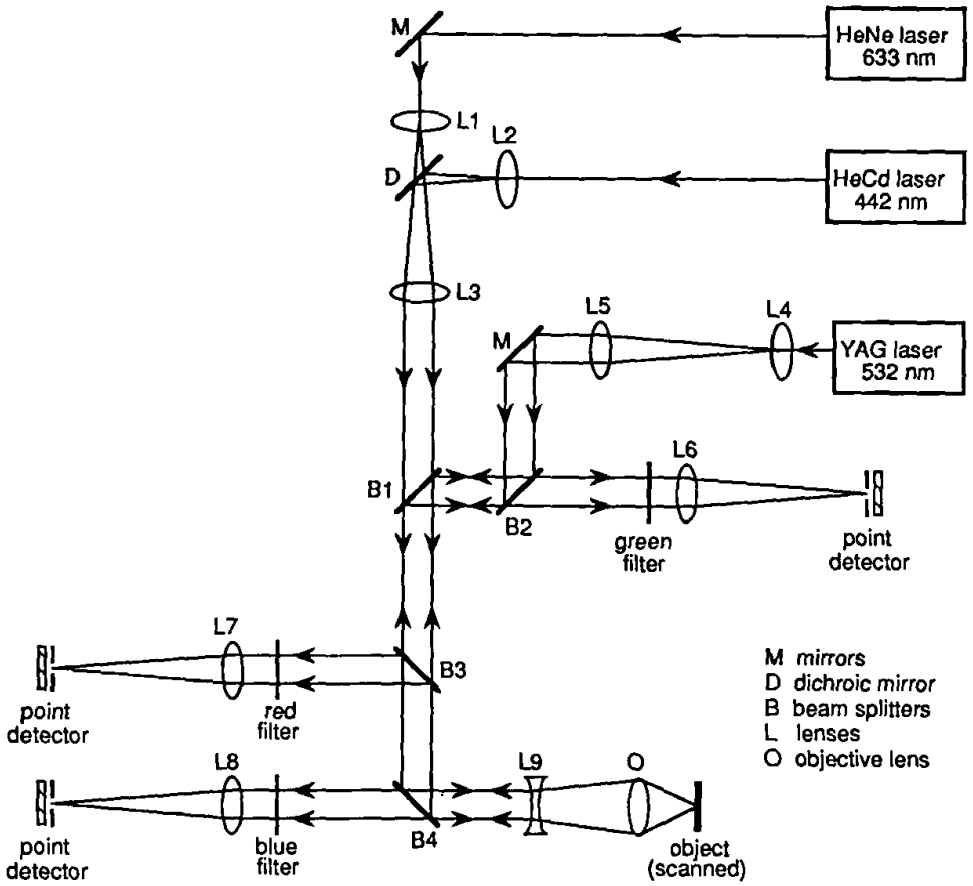

Fig. 2. The optical arrangement of the three-colour confocal scanning optical microscope.

object is thus suppressed, rather than appearing blurred as happens in the conventional microscope. The optical-sectioning property is usually characterized by the so-called defocus response. (This is sometimes referred to as the $V(z)$ response which is the analogous technique in acoustic microscopy.) The defocus response is measured with a perfect reflector as object, and gives the variation of the detector intensity output as the object is scanned axially through focus. A typical defocus response curve is shown in Fig. 1(c) with intensity (I) plotted along the vertical axis and the focus position of the objective (z position) along the horizontal axis. The two z positions indicated on this plot correspond to the focus positions in Fig. 1(a, b). A standard method for evaluating performance using the defocus response technique is to measure the full width of the peak at half the maximum signal intensity (FWHM). For example, with red light and an objective lens with a numerical aperture of 1·3, the defocus response typically has a FWHM value of less than 0·5 μm which is a reasonable indicator of the axial resolution obtained using this configuration.

A THREE-COLOUR CONFOCAL REFLECTION MICROSCOPE

Experimental apparatus

The optical arrangement of the three-colour confocal microscope is shown in Fig. 2. The laser light sources are HeNe: 633 nm; frequency-doubled YAG: 532 nm and HeCd: 442 nm. Beam expansion and collimation are achieved for the red light by lenses L1 and L3 (adjusted to give an accurately parallel beam), for the blue light by L2 and L3 and for the green light by L4 and L5. Lenses L2 and L4, which are low-power microscope objectives, are mounted so that their axial and transverse positions with respect to the optical axis are adjustable. The red and blue beams are combined with minimal power loss by the dichroic mirror D, and the green beam is introduced via the beam splitter B1 which also passes part of the reflected beam to one of the detectors

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