SHORT TECHNICAL NOTE

New imaging modes for lenslet-array tandem scanning microscopes

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Summary

The tandem scanning microscope permits confocal images to be obtained in real time and viewed directly by eye. The light budget of these instruments may be increased from a few percent to a few tens of percent by incorporating an array of microlenses so as to increase the amount of illumination light that reaches the specimen. These instruments are configured for fluorescence imaging together with laser illumination. We describe how the versatility of the instrument may be enhanced to permit the use of incoherent light sources as well as extending the imaging modes to include bright-field reflection.

Introduction

Scanning in confocal microscopes can be achieved in a variety of ways, and a particularly attractive configuration for real time imaging is the tandem scanning microscope (TSM) (Petran *et al.*, 1968; Boyde *et al.*, 1990). In these instruments the illumination and detection pinhole arrays are placed diametrically opposite on a spinning Nipkow disc. A subsequent development permitted the same pinhole array to be used for both illumination and detection (Xiao *et al.*, 1988). A recent development to this 'single-sided' TSM has been the introduction, by the Yokogawa company (Japan), of a lenslet array to permit more efficient use of the illumination light. The manufacturer's literature claims light transmission efficiencies of the disc of up to 60% when used for fluorescence imaging using coherent illumination sources. Although fluorescence imaging is all but ubiquitous in biological confocal microscopy, there are major benefits to be gained from back-scattered or reflected light imaging when examining samples such as oral soft and hard tissues and dental biomaterials (Watson &

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Boyde, 1991). To enhance the capabilities of the lenslet array TSM to permit reflected light imaging we describe a possible configuration of the Yokogawa CSU10, a lenslet array TSM (Ultraview, Perkin Elmer, Cambridge, U.K.).

These instruments work well in fluorescence, with easy discrimination between the excitation and emitted light provided by the dichroic mirrors located between the Nipkow disc and microlens array (Fig. 1). There is therefore little problem with unwanted reflected light from optical components within the scanning system. The aim of this study was to determine whether, via a simple modification, non-laser incoherent illumination could be used to advantage.

Reflected laser light imaging

It is possible to produce reflected light imaging using the standard laser illumination of this system by using a multipass dichroic set, with 'incorrect' barrier filters in place. As with all single-sided TSMs, the illuminating and reflected light can be separated by using crossed polarizing filters, with a suitably positioned quarter wave plate. This will remove many of the unwanted back reflections from within the optical path but reduces the intensity of the image signal. The extent of the back reflections is variable, depending upon the objectives in use. Most of the interfering signal is probably derived from the scanning disc surfaces, which in other single-sided TSMs would be slightly tipped relative to the illuminating beam, so sending this unwanted reflection off-axis to a light trap (Xiao *et al.*, 1988). Laser illumination can be used for the microscope, but there is a risk of direct viewing by eye of the reflected laser light, through the eyepieces, leading to significant safety issues.

Use of incoherent light sources

To overcome these concerns and increase the operational flexibility of the microscope we have used incoherent white-light
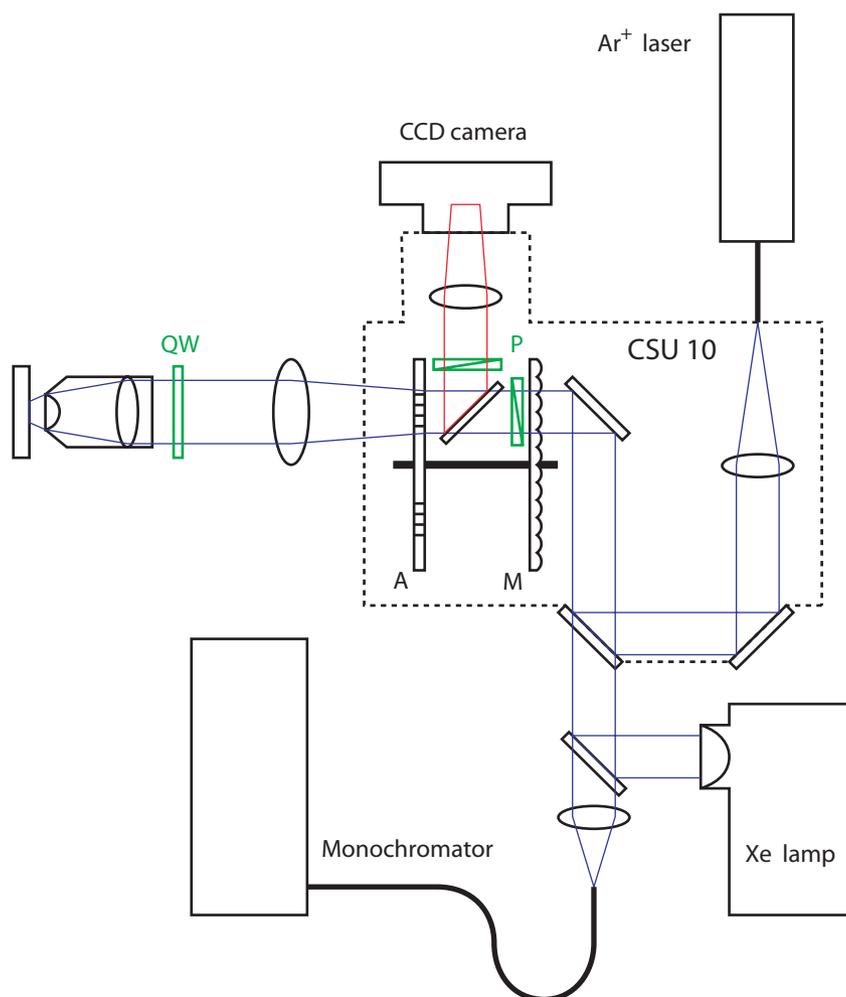
Fig. 1. Schematic arrangement of the optical system showing the various illumination sources. The quarter wave plate (QW) and the two linear polars (P) required for operation in reflected light are shown in green. The pinhole aperture disc (A) and the microlens array (M) are also shown.

illumination sources. These have consisted of a 250 W xenon arc source in a conventional lamp housing or a small arc 100 W xenon lamp in a monochromator format (Spectra-MASTER 2: Perkin Elmer). The light source was projected into the instrument as close to the scanning discs as possible, bypassing the collimator optics. Polarizing filters and a quarter wave plate were used, as before (Fig. 1).

Experimental procedures

In order to demonstrate the effectiveness of this modification, both samples of tooth restored with fluorescent labelled filling materials and autofluorescent pollen grains were imaged. The effect of spectral bandwidth of the source was examined by using a xenon arc lamp unfiltered. A monochromator was used to produce essentially single wavelength illumination at 485 nm, so mimicking the laser. This was configured as shown in Fig. 1.

We did not use the host microscope but rather a combination of a 10-cm tube lens together with a range of infinity corrected microscope objective lenses (Olympus) (Fig. 1). This

also served to increase the light efficiency as well as to double the field of view, which in the original configuration is limited to 7×10 mm in the intermediate image plane. The images were recorded with a cooled CCD camera (KAI310: Perkin Elmer) noting the operating conditions and sensitivity settings of the camera. The optical sectioning capabilities of the instrument were determined by extracting the axial response from the images recorded of a mirror tilted such that the bright band – which characterizes the optical sectioning property of the microscope – was clearly visible within the field of view. The axial response was also measured with 488 nm (laser illumination), white light and incoherent illumination at 485 nm wavelength.

Results

The images of the dental samples showing tooth–restoration interfaces can be seen in Figs 2–5, with reflected light and fluorescence images. It is noteworthy that the reflected light images provide valuable detail such as the dentine tubules and voids within the restoration interface (arrow, Fig. 4), features
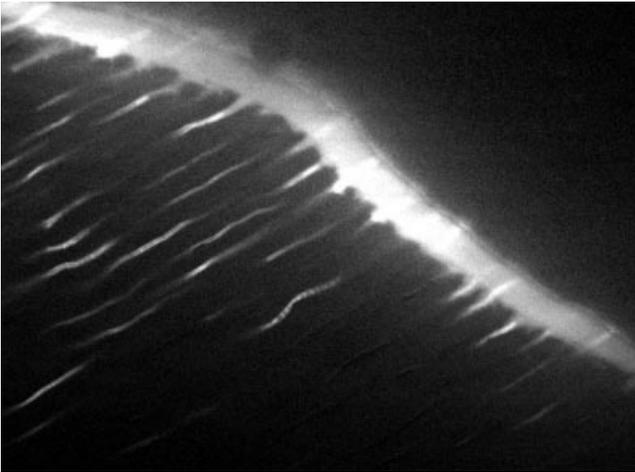
Fig. 2. Fluorescence image of dentine / composite adhesive restoration interface. The adhesive was labelled with fluorescein. 488 nm laser illumination. The objective used was 60×, 1.4 NA oil immersion lens. Field width 200 μm.
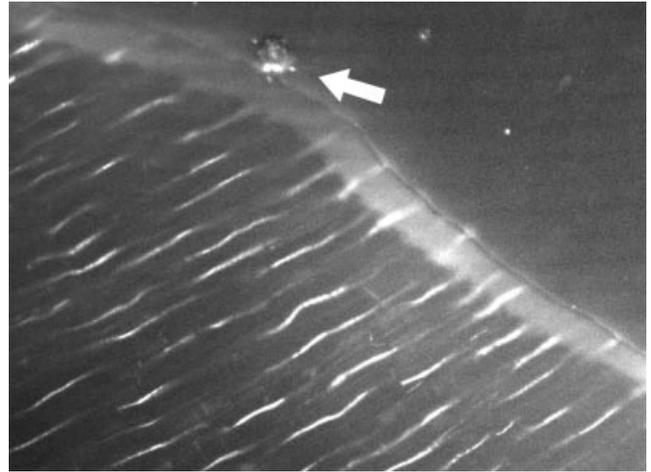
Fig. 4. Same field of view as Fig. 2 but reflected light image with full spectrum illumination. Note that dentine tubules are now visible, with an air bubble at the middle-top of the adhesive layer (arrowed).
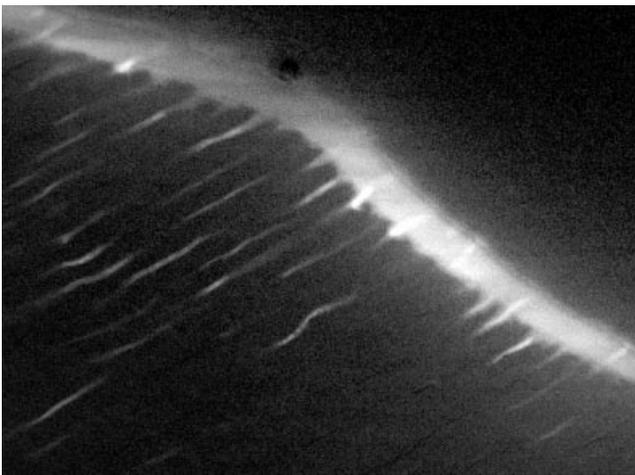
Fig. 3. Same field of view as in Fig. 2. but illuminated with 485 nm incoherent light source from a xenon lamp. Note the circular defect visible in the adhesive layer.
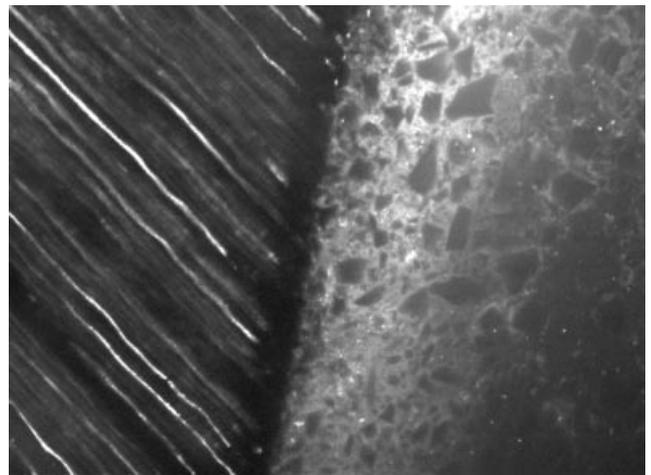
Fig. 5. Glass-ionomer cement / dentine interface. Combined reflection/ fluorescence image with full spectrum illumination. No information would have been derived from the dentine if fluorescence imaging mode had been used in isolation. The objective used was 60×, 1.4 NA oil immersion lens.

that are not apparent from the fluorescent image alone. Figures 6 and 7 show fluorescence images of pollen grains illuminated with coherent and incoherent light sources. Image quality was comparable between the two types of illumination source, but there were differences in signal intensity. The camera gain needed to be increased by a factor of 10 in the incoherent illumination when compared to laser illumination.

Figure 8 shows the axial responses measured using a variety of light sources and a 60×, 1.4 NA objective lens. The traces are offset vertically to ease viewing and comparison. As can be seen, the responses are fairly similar and show a full-width-half-maximum width of around 1 μm. The response obtained with the blue laser line (488 nm) indicates the

presence of residual spherical aberration in the system, as can be deduced from the asymmetry in the curve. The very slight 'ringing', which can be observed in the wings of the response, can probably be attributed to the interference between the light beams transmitted through adjacent pinholes. The white light response, by contrast, is slightly wider, as would be expected, as it represents the superposition of responses at many wavelengths including the long wavelength (red) tail, which inevitably broadens the response. The exact response in this case is difficult to predict as it depends on both the spectral properties of the source and the spectral sensitivity of the camera.
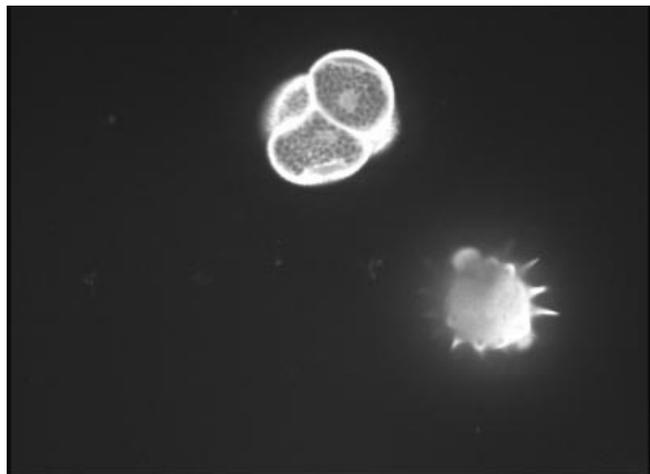
Fig. 6. Pollen grain imaged with 488 nm illumination. A 100 ×/ 1.4 NA oil immersion lens was used in this case. Field width 120 μm.
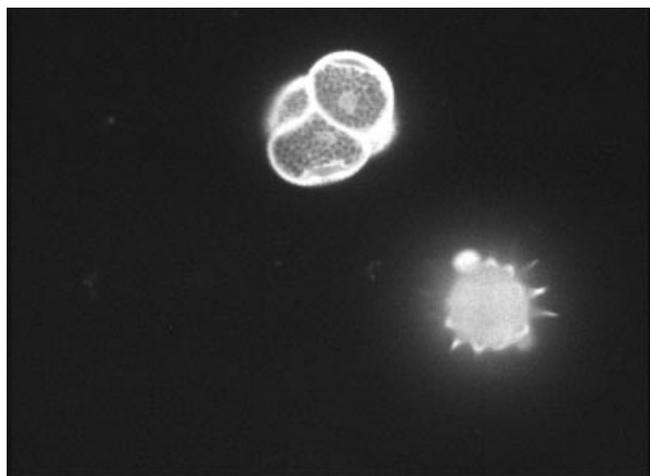

Fig. 7. Similar field of view to Fig. 6 but using 485 nm line illumination from the monochromator.

Conclusions

We have demonstrated a simple modification that permits incoherent light to be introduced to a lenslet array TSM. The optical sectioning characteristics of the instrument are not dramatically affected by using incoherent illumination. However, there is a very significant reduction in the fluorescence image signal level when compared with laser illumination. Indeed, this reduction, which can amount to more than an order of magnitude, is readily explained by the operation of the lenslet array. Laser illumination, which is effectively a point source, can be focused by the individual lenses in the array to almost diffraction-limited spots which coincide with the pinhole apertures in the second (pinhole) disc, and thereby result in relatively high transmission efficiencies. By contrast, with a finite sized illumination source (Arc lamp) this is not the case,
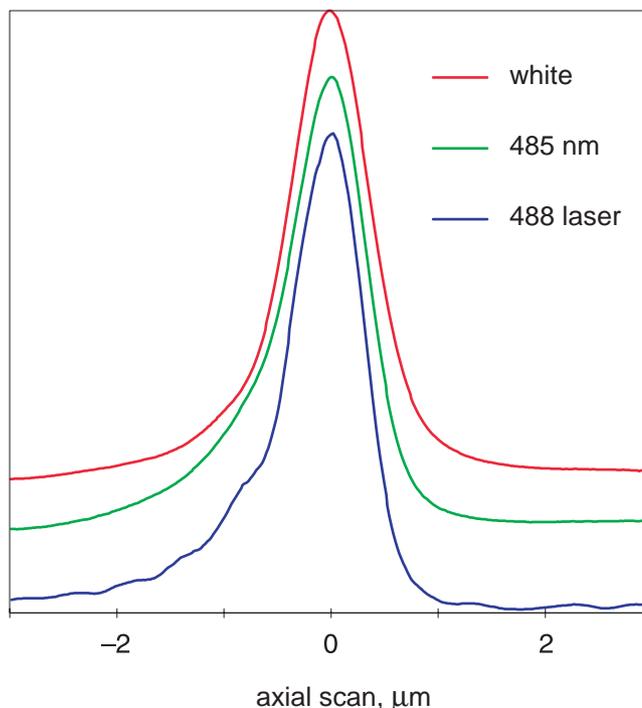

Fig. 8. Axial responses of the microscope obtained with different light sources with a 60×, 1.4 NA microscope objective. The traces are offset vertically to ease viewing and comparison.

as the illumination on the pinhole disc is more or less uniform despite the presence of the lenslet array. Therefore, the transmission efficiency is determined by the pinhole array alone and hence is similar to that obtained in traditional Nipkow disc systems, i.e. about 4% for the particular disc used. A simple explanation of this fact can be obtained by considering the individual images of the light source created by the lenslets on the pinhole disc. These images are found to be much larger than the pinholes, and actually overlap significantly, and therefore lead to largely uniform illumination. This is a fundamental consequence of the incoherent nature of the illumination source. However, despite this reduced light budget, we have shown that safe direct viewing of high quality reflected light images is possible.

References

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