SHORT COMMUNICATION

Wide-field optically sectioning fluorescence microscopy with laser illumination

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Summary
We describe an extremely simple method by which optically sectioned fluorescence images may be obtained with conventional microscopes using laser illumination. A one-dimensional grid pattern is introduced into the illumination system, together with a rotating ground glass diffuser. This causes an image of the grid pattern to be projected into the specimen. Images taken at three spatial positions of the grid are processed in a simple manner to provide optically sectioned images of fluorescent specimens.

The use of the confocal microscope to image volume structures in three dimensions is a widely used technique in many fields of science and particularly in the biosciences. This is possible because, unlike the conventional light microscope, the confocal microscope possesses an optical sectioning or depth discrimination property which restricts the portions of a volume object which are imaged efficiently to those lying close to the focal region. The confocal optical system achieves this by using a point detector, which physically prevents light from out-of-focus planes contributing significantly to the final image (Wilson & Sheppard, 1984; Pawley, 1995). A volume rendering of an object may be created by sequentially recording a series of through-focus images followed by appropriate computer processing.

An alternative method of producing optically sectioned images in real-time in a wide-field microscope has recently been proposed (Neil et al., 1997; Wilson et al., 1998; Lanni & Wilson, 1999). This method, which requires minimal modification of a standard wide-field conventional microscope, has been used to obtain high quality images using standard, non-laser, microscope light sources. The basis of the method is to project an image of a one-dimensional grid onto the specimen. Images, taken at three spatial positions of grid projection, are then processed in real-time to produce the optically sectioned image. Although this approach is satisfactory for many imaging applications, there are cases when laser illumination may be preferable. One example would be when illumination at a specific wavelength is required. Although this wavelength may well be available from a white light source, it will be considerably weaker in intensity than that obtainable from a laser. Another example would be fluorescence lifetime imaging, where it is necessary either to illuminate the sample with a very short pulse of light and analyse the time decay of the fluorescence emission or to amplitude modulate the excitation light and to analyse the phase shift between it and the fluorescence emission in order to calculate fluorescence lifetimes (Lakowicz & Berndt, 1991; Morgan et al., 1995; Carlsson & Liljeborg, 1997; Schneider & Clegg, 1997). We will now show that laser illumination can also be used with this new approach which opens up the possibility of, for example, performing wide-field three-dimensional lifetime imaging with minimal modification to a conventional system.

Figure 1 shows the optical system used to project an image of a one-dimensional line pattern (25 μm pitch) onto the object. This was achieved using the telescope system as shown so that no internal modifications were required to the host Zeiss Axiovert 135TV microscope. In order to eliminate the effects of laser speckle a rotating ground-glass disc was introduced into the illumination system in close proximity to the grid. An argon/krypton laser (Coherent...
Innova 70C Spectrum) was used as the light source, together with a scientific grade cooled CCD camera (Photometrics Quaintix) incorporating a 12-bit chip (Kodak KAF1400, Grade 1) to capture the raw images.

The fundamental basis of the method to obtain an optically sectioned image from this system has been described in detail elsewhere (Neil et al., 1997) and so we will merely state here that it is necessary to capture three images corresponding to a relative spatial phase shift of the grid of 120° between images. This spatial phase shift is accomplished by physically moving the grid laterally with a piezoelectric positioner. The sectioned image is then obtained by calculating the square root of the sum of the squares of the differences between each of the three images. The conventional image may also be recovered merely by adding the three raw images. In this way it is possible to display an optically sectioned image and a conventional image simultaneously (Neil et al., 1997).

Figure 2 shows a through-focus series of images of COS-7 cells. The images were taken using 514 nm excitation and a 100× 1.4 NA oil immersion objective. This lens, together with the external optical system, caused the grid pitch in the object plane to be 1.2 μm. The sectioned images, taken at 2 μm focal settings, are shown on the left of the figure, together with the corresponding conventional image on the right. Both series of images, which show the distribution of the calcium/lipid-dependent protein kinase Ca (PKCa) in fixed COS-7 cells, were calculated from three raw images as indicated above. The contrast in the images was achieved by immunostaining with Cy3 fluorescently labelled MC5 Fabs (Ng et al., 1999). The quality of the optical sections produced can be clearly seen in the figure.

In conclusion, we have shown that high quality optically sectioned fluorescence images may be obtained using laser illumination in a wide-field conventional microscope with relatively minor modifications to the illumination system. The approach we have described has the added benefit of providing a conventional image simultaneously with the optically sectioned image.

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Fig. 2. A series of fluorescence images taken at 2 μm intervals. The sectioned images are shown on the left and the conventional images are shown on the right. The scale bar represents 10 μm.
References


