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Multi-colour Confocal Microscopy by Means of Intensity-Modulated Multiple-beam Scanning (IMS)

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For the analysis of the spatial relationship between different biological structures in the same preparation, dual-colour confocal microscopy (Carlsson, 1990) has proven to be a successful technique. For example: experiments where DNA double labelling techniques were combined with accurate distance measurements in dual-colour confocal images have shown that DNA replication is a dynamic process (Manders et al. 1992, Manders et al. submitted) . However, it is impossible to prove the existence DNA move-ment during the replication process by using a confocal microscope equipped for the detection of only two colours. A third (FISH; fluorescent *in situ* hybridization) label should be used to investigate the DNA-replication process in more detail. Also, in many other biological and biomedical applications the recording of more than two different fluorophores in the same preparation (Multi-colour confocal microscopy) is an urgent need. Therefore, we built a confocal microscpe for the voxel-simultaneous detection of three or four fluorophores.

Major interfering effects in multi-colour confocal micrscopy (Brelje et al. 1993) are: 1) *cross-talk* between the fluorescence signals and 2) positional *shift* between the colour components of an image. To minimize the latter effect, all fluorescence signals should be recorded during one scan (exact repositioning of the scanning table is difficult) and UV-excitation of one of the fluorophores should be avoided (large differences of excitation wavelengths will generally spoil the alignment of the fluorescence signals). To minimize the amount of cross-talk we applied a new technology, Intensity-modulated Multiple-beam Scanning (IMS) (Carlsson et al. 1994), that drastically reduces the cross-talk between the fluorescence signals.

In this study we investigated the possibility to use the fluorophores FITC, Texas Red and Cy-5 for voxel-simultaneous scanning. For the excitation of these fluorochromes we used an Argon laser tuned to 488 nm and a Krypton laser in multi-line mode. From the Kr-laser the wavelengths 568 nm and 647 nm were selected and subsequently separated (Fig. 1). The three wavelengths predominantly excite one fluorophore each. The three laser beams were intensity-modulated by three electro-optical modulators (EOMs) tuned to different frequencies in the MHz region, and combined before entering the confocal microscope. The emitted light was spectrally separated and detection was performed by three photomultiplier tubes (PMTs) with filters for green (500-550 nm), red (580-630 nm) and far-red (665-695 nm). The outputs of the PMTs were connected to three lock-in amplifiers, each tuned to the modulation frequency of the corre-sponding laser beam. The signals from the three lock-in amplifiers were sampled voxel-simultaneously and subsequently digitized and stored. The recorded (full-colour) images can be displayed during scanning.

In our experiments we used preparations of pure fluorochromes, fluorochrome-conjugates and microbeads coated with fluorochromes. All measurements were performed with, and without the lock-in detection technique in order to determine the effect of the lock-in technique on cross-talk quantities. In addition to the described triple-colour set-up, we have investigated the use of a fourth fluorophore, without using an additional laser-line or an additional PMT for this purpose we tested the excitation and emission quantities of PerCP (Beckton Dickinson). This fluorophore can be excited with 488 nm and emits in the far-red region. By using an extra lock-in amplifier connected to the 'far-red PMT' and tuned at the modulation frequency of the 488-nm-beam, we managed to separate the PerCP-fluorescence signal from other

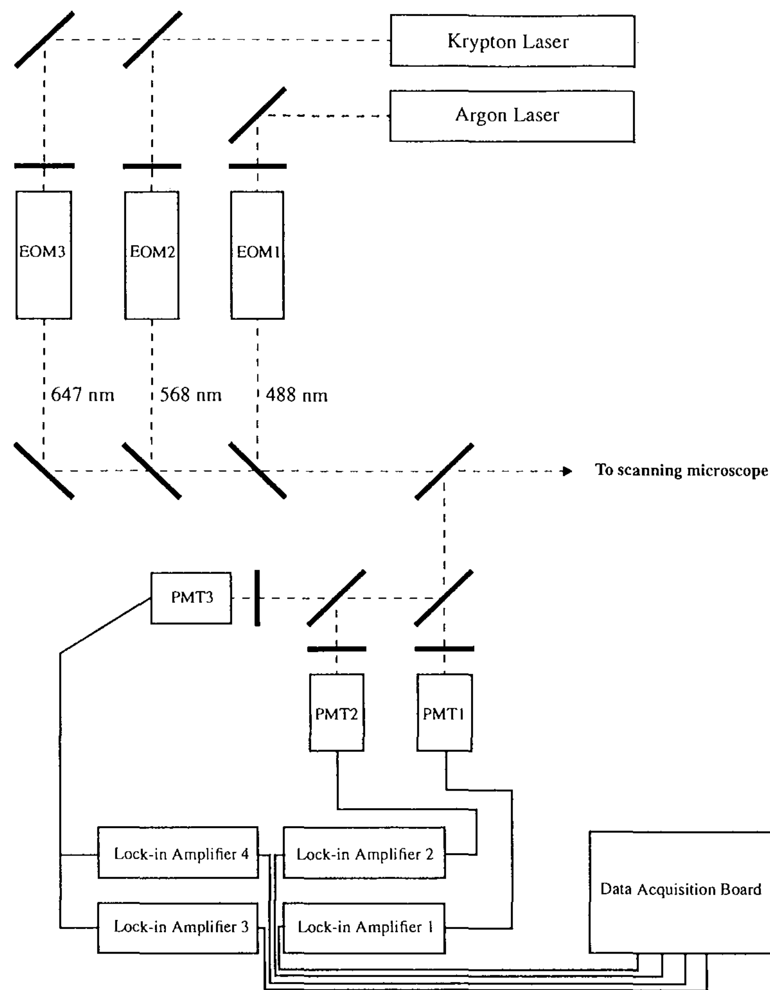

Fig.1. The three colour IMS set-up. As shown, the set-up is con-figured for scanning four fluorophores using two lasers three detectors and four lock-in amplifiers.

fluorescence signals. In this manner, the IMS technique can be used for the application of an extra fluorophore (instead of cross-talk reduction).

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