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Confocal Microscopy with Electronic Pinholes

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PROBLEMS IN BIOLOGICAL CONFOCAL MICROSCOPY

Especially in the biomedical field, the accurate imaging of tiny three-dimensional structures and the measurement of time-evolving microscopical phenomena, generally require that the specimen is irradiated in a spectrally adequate and non-invasive way. Unfortunately, current confocal microscopes give their best results in materials research applications on reflecting specimens in which spectral requirements, irradiation damage and saturation are of minor or no importance.

Actually, the design of most confocal microscopes, is based on the use of laser sources in a confocal-point (CP) scanning, epi-illumination configuration. On one hand, in fluorescence analysis, this imposes scarce flexibility and relatively high costs in the selection of a source being suited to a particular chromatophore. On the other hand, in many cases, specimen damage due to the high instantaneous irradiation doses and fluorescence saturation effects can't be tolerated (Wells et al. 1990, Visscher et al. 1994). Moreover, instrumentation in the latter class is substantially unsuited to transmitted-light confocal analysis that is very important in many branches of the biological and medical investigation.

In order to overcome some of the cited limitations, confocal microscopes have been proposed, based on multi-confocal-points (MCP) scanning approaches, with the use of revolving spatial modulators (Egger and Petran 1967, Kino and Xiao 1990, Lichtman and Sunderland 1989) or on confocal-line (CL) illumination and detection methods (Benedetti et al. 1992, Brakenhoff and Visscher 1992). Conventional sources can be more favorably adopted in these cases and the instantaneous irradiation dose on the sample is substantially reduced, due to parallel operation. When observing specimens of small or moderate thickness, the spatial resolution performance of MCP instruments essentially corresponds to that of CP instruments, but unfortunately the construction is even more complicate and costly, the versatility is scarce and the epi-illumination configuration prevents any practical possibilities for the study of the light-absorbing properties of the specimens. On the other hand, the CL methods existing offer interesting capabilities, but their spatial resolution performance remains inferior to that of CP and MCP techniques (Benedetti et al. 1994, Sheppard et al. 1991).

OPTICAL OR ELECTRONIC FILTERING ?

In current confocal instrumentation, most of the light available from the specimen is deliberately thrown out (Wells et al. 1990) by the spatial selectivity of the detection pinhole (optical filtering). Consequently, in many applications, such as in the detection of low fluorescence signals but even in the case of three-dimensional reconstructions (Hiraoka et al. 1990), an extensive part of microscopical activity is presently performed with the aid of non-confocal microscopes, using high performance image sensors and computer deconvolution of spatial data (electronic filtering). While affected by intrinsic limitations in the ultimate resolution performance, if compared to the confocal techniques (Frieden 1967, Streibl 1985, Carringhton et al. 1990, Sandison and Webb 1994), the latter approach can be more sensitive and offer a higher signal-to-noise ratio, along with a higher biological compatibility. Also, the experimental setup is more adaptable to a variety of microscopies and different analytical requirements, due to the

use of the conventional instrument, associated to an advanced image sensor with relatively powerful data processing devices and consequent programming flexibility.

A MORE RADICAL SOLUTION

As a further improvement to the performance of the current methods discussed above, the authors have devised and experimentally implemented a novel microscopical imaging technique aimed to combine advantages of confocal and non-confocal microscopies, called Electronic Multi Confocal Points (EMCP) microscopy (Benedetti et al. 1995). The method is based on a substantially unmodified microscope design and can achieve conventional, partly-confocal or confocal imaging performance in several configurations including reflection, fluorescence, transmission, etc. Any imaging activity is performed with the aid of an image sensor fitted to the photographic tube of the microscope (Fig. 1).

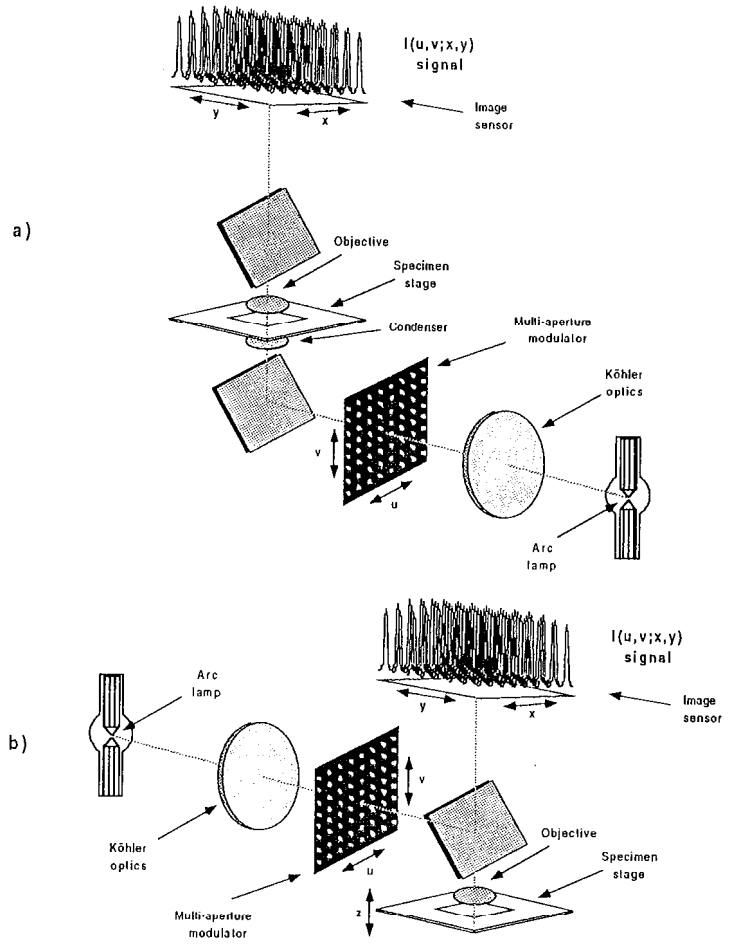

Fig.1. The EMCP optical setup. a) transmission configuration, b) epi-illumination configuartion

In cases in which the confocal imaging performance is desired, a multi-point spatial modulator (hole-pattern) is placed in an image plane along the illumination path of the microscope. As a consequence, the specimen is Koehler illuminated by the multitude of spots that corresponds to the demagnified pattern of the modulator. The image sensor is equally used for the detection, but a spatial filtering process, conjugated to the illumination spots, is performed here at the electronic level, selecting only those pixels that correspond to the illumination spots. Spot positioning, on the detector surface, is obtained during a preliminary reference measurement. A set of subsequent images is collected while the modulator is scanned in its plane in steps capable of covering the full object by means of interleaved spot positions. The summation of the images collected in this way, represents the confocal image of the object.

The method is closely related to other MCP techniques, with the important difference that critical opto-mechanical parts are substituted by more reliable opto-electronic processes, taking advantage of modern technology.

The optical and mechanical characteristics of the spatial modulator are not particularly demanding in EMCP and the size of the illumination spots, their distribution and the fill-factor can be easily changed with the simple substitution of the modulator itself. The latter capability is very important to optimize the multi-point operation to the specific characteristics of the sample. The initial reference measurement provides to adapt the data collection process to the characteristics of the particular modulator selected and also to correct a relatively wide class of imperfections due to modulator manufacturing.

CONCLUSIONS

The EMCP method is simple and the resulting instrumentation, compared to existing confocal systems, is relatively simplified since it requires only modest opto-mechanical additions to a normal research microscope and an accurate image sensor followed by suitable components for electronic data processing.

As a result, the most salient features of EMCP microscopy are:

a) The instantaneous irradiation doses are orders of magnitude less than in CP microscopy, resulting in lower specimen damage, improved spatial resolution and linear photometric characteristics. The latter aspect is specially important when spatial deconvolution procedures are employed on image data.

b) Conventional light sources can be used with substantial advantages in spectral flexibility, simplified design and low cost.

c) The method is adaptable to different microscopies and offers improved peculiarities, over existing approaches, in the measurement of space and time dependent phenomena.

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