Programmable Array Microscopes

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The programmable array microscope (PAM) is a powerful tool combining the capabilities of nearly all previously described optical sectioning techniques in a single microscope. Not only can the user create optical sections of three-dimensional objects, but the PAM's unique adaptive optical strategy allows a user to select the best sectioning method for a particular sample or experimental need. The key to the PAM is a spatial light modulator (SLM). This device, when placed in the image plane of a microscope, can be used to create optical sectioning, generate spatial encoding masks, and/or define regions of interest. The growing family of PAMs includes optical sectioning systems operating in fluorescence[4,3,7,1,12] and reflection[7,1,12], as well as spectroscopic imaging systems for fluorescence emission spectroscopy. Perhaps the most common application of this unique family of adaptive optical systems is optical sectioning.
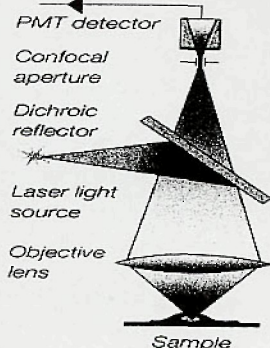
Optical sectioning microscopes typically use single point, line, or rotating disk scanning (Figure 1). The rotating disk scanning systems normally consist of repeating patterns of dot or line arrays lithographically produced on the surface of a disk. Recently, disk systems utilizing random bit sequences[6,16] or regularly spaced apertures covered with microlenses have come into use. These modifications increase the optical throughput of the rotating disk and hence the speed of the measurement. Such microscopes have limitations when observing thick specimens and, since they are not made with adaptive components, are typically optimised for a particular magnification. The degree of optical sectioning depends on the periodic structure of the pattern, the numerical aperture of the objective, the wavelength of light, and the magnification.[5,15.] In this context, the programmable array microscope is advantageous since it allows most scanning methods to be reproduced under programmable control and the scan pattern can be adapted to best match a particular sample and lens.

The core of any programmable array microscope is a spatial light modulator placed in the primary image plane of the microscope. Spatial light modulators come in a variety of types and formats, with digital micromirror devices (DMDs) and liquid crystal devices (LCD) being the most common (Figure 2). Both technologies have been extensively developed to service the needs of video projector systems. As such, they are available in mass quantities at reasonable cost. The use of a DMD in optical sectioning arrangements provides the unique capability to select the non-conjugate (out-of-focus) light, which is normally lost at the pinhole in a confocal microscope, and send it along a separate optical pathway (Figure 1c).[5]

A second application in which an adaptive optical element is useful is for generating spatial encoding masks for imaging spectroscopy[5]. There are three standard ways in which two-dimensional spectral images are acquired in microscopes. These consist of point[10], slit scanning[13], or wavelength scanning (Figure 3)[11]. These three methods describe the bulk of imaging spectroscopy done in microscopes. Wavelength scanning currently is the most popular of the methods shown and has been implemented with variable interference filters[17], acousto-optical tuneable filters[9], and liquid crystal tunable filters[9]. In situations where only a limited area of the sample is of interest or a limited number of wavelengths are needed, these filter types remain the methods of choice. However, they share a common disadvantage in that the majority of the light is rejected or the majority of the sample is outside the region of view. When large areas of a sample are of interest, multiplexed methods allow a greater amount of the sample to be observed simultaneously. Fourier encoding of the wavelength

Signal Amplifier

PMT detector
Confocal aperture
Dichroic reflector
Laser light source
Objective lens
Sample

Confocal Microscopy Optical Layout
Figure 1A

Laser
Rotation
Microlens Array
Microlens
Lens
Camera
Pinhole
Objective lens
Specimen

Figure 1B

Camera A
Excitation Source A
Excitation Source C
Camera B
Excitation Source B
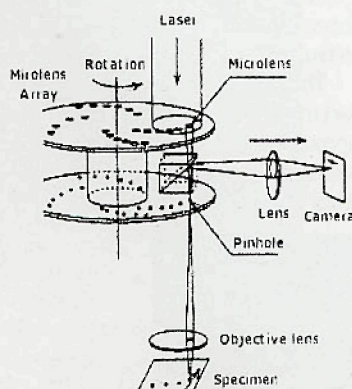
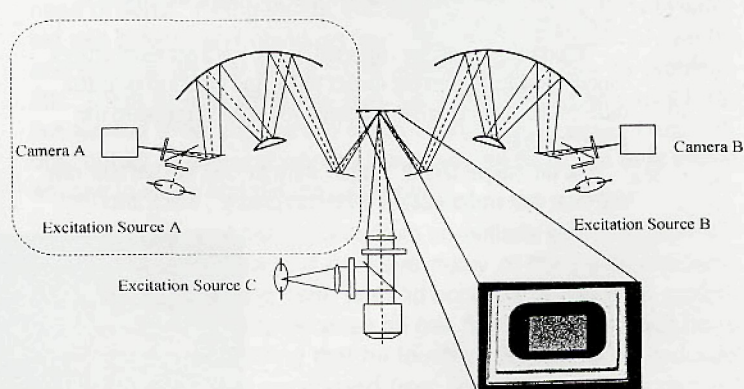**Figure 1C**

Figure 1 Confocal Scanning systems. The simplest and still most common optical sectioning microscope is the confocal laser scanning microscope (A). Here the scanning is done with the focused spot of a laser beam and out of focus light is removed at the confocal aperture. A variation on this approach is to use a line of illumination and slit detection. Another approach to confocal scanning is the use of regularly spaced apertures arranged on the surface of a rotating disk (B). This allows many apertures to scan the sample simultaneously. The system shown illustrates the use of microlenses to improve the utilization of illumination light. The disadvantage of the rotating disk is that the pattern of illumination and detection cannot be adjusted to for a particular purpose. This problem is solved elegantly by the programmable array microscope (C). The PAM can uses the elements of a spatial light modulator to scan a specimen. This allows the PAM to do point and line scans and to adapt the spacing of the pattern and the aperture size to a particular experimental need. The system shown illustrates acquisition of separate conjugate, I_c ("in-focus"), and non-conjugate, I_{nc} ("out-of-focus"), images. When excitation source A is activated, the "in-focus" image forms on camera A while an "out-of-focus" image forms on camera B. Conversely, if excitation source B is activated, the positions of I_c and I_{nc} are reversed. The area surrounded by the dotted lines shows a single sided segment. The two-sided arrangement is currently under development.

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domain has proved successful for generating two-dimensional spectral images[8]. Hadamard transform techniques, however, have a number of special advantages for this type of measurement. For example, when implemented with stationary mask technology such as a liquid crystal SLM, no moving parts are required. Also the optics are relatively simple and do not require exceptional care to align. In contrast, the patterns used in mask lithography, such as used to produce rotating disks, can not be rapidly adapted to meet the needs of a particular specimen or purpose under programmable control. However through the use of spatial light modulators, pixel element sizes can be adjusted for rapid screening at low resolutions or careful examination of higher resolution structures.

Optical sectioning programmable array microscopes

An optical sectioning PAM was implemented as an add-on module to a Nikon E-600 microscope. To define patterns of illumination and detection, a Texas Instruments DMD (Plano, TX) was mounted in the primary image plane and used. The light from the "on" elements was relayed to an Apogee KX-2 charge-coupled device (CCD) camera (Apogee Instruments, Tucson, AZ). The quality of the optical relay was sufficient to observe effects from the 1 μm dark segments between adjacent DMD elements. The DMD was illuminated through a Nikon filter block using a 250 W super high pressure Hg arc lamp system (Lumatec GmbH, Munich, Germany), a 450 W Xe arc lamp (Müller Elektronik-Optik, Moosinning, Germany), or an argon ion laser (Coherent, Santa Clara, CA). Axial positioning of the objective was accomplished using a PIFOC piezoelectric system with a resolution of 10 nm (Physik Instrumente, Waldbronn, Germany). A block diagram of the microscope and add-on are given in Figure 1C.
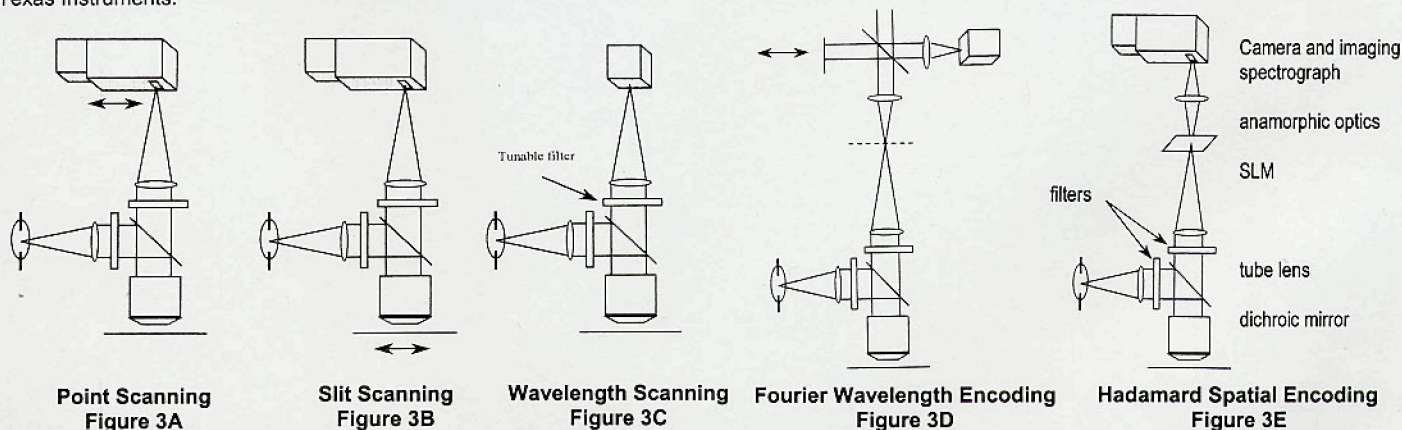
Spectroscopic PAM

A fluorescence Hadamard transform programmable array microscope (PAM) is as shown in Figure 3E[3]. The system consisted of an add-on to a Nikon E-600 microscope equipped with a 100 W Hg arc lamp and an epi-fluorescence unit. This spectroscopy module consisted of a SVGA format LCD device SLM (Central Research Labs, England), anamorphic relay optics, a PARISS prism-based imaging spectrograph (Lightform Inc., Belle Mead, NJ), and a KX-2 CCD camera (Apogee Instruments, Tucson, AZ). The pixels of the SLM consisted of an irregular element approximating a 26 μm (horizontal) by 24 μm (vertical) rectangle reproduced on a 33 μm square pitch. The active element was a TFT twisted nematic LC array in an 800x600 format. The SLM was used to display a series of bar patterns defined by the rows of cyclic S-matrices. An image was recorded for every row of the S-matrix. After subsequent decoding with an inverse S-transform, a three-dimensional image stack was obtained.

CRL LCD
800 x 600
33 μm pixels

TI DMD
848 x 600
17 μm pixels
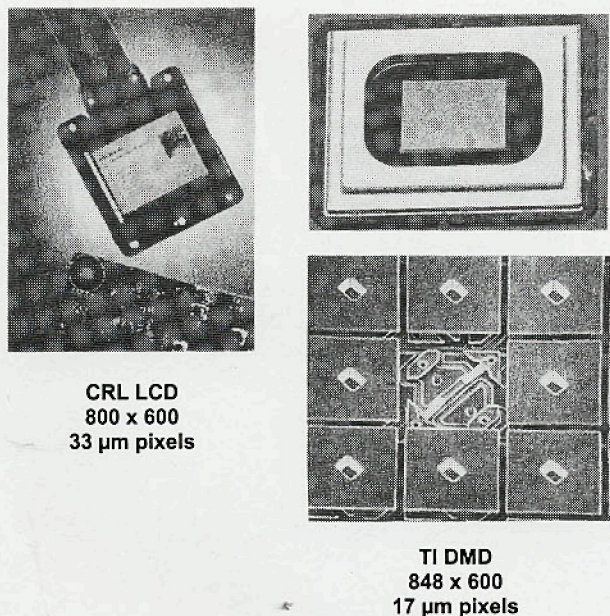
Figure 2 Two spatial light modulators used in PAMs. The SLM on the right is a transmission liquid crystal device provided by CRL laboratories. The SLM device is a digital micromirror device (DMD) made by Texas Instruments.

Point Scanning
Figure 3A

Slit Scanning
Figure 3B

Tunable filter

Wavelength Scanning
Figure 3C

Fourier Wavelength Encoding
Figure 3D

Camera and imaging spectrograph

anamorphic optics

SLM

filters

tube lens

dichroic mirror

Hadamard Spatial Encoding
Figure 3E

Figure 3 Spectroscopic imaging methods. The most common are point (a), line (b), and wavelength scanning (c). Point scanning systems look at a single point in the image field and record the spectrum after passing with a scanning monochromator or spectrograph. Observing line transects can increase the speed of acquisition of the point scanning approach. In the latter approach, a camera is used to detect a two dimensional image consisting of one spatial axis and wavelength. The two dimensional spectral image is then acquired by recording a series of such images at different spatial locations. For imaging a small number of wavelengths, the use of a wavelength tunable filter and CCD camera detection is one of the most efficient. These methods share a common disadvantage in that most of the sample or the range of available wavelengths is not available for observation. To avoid these limitations, multiplexed methods have been developed using both Fourier (d) and Hadamard encoding techniques (e). The latter is well adapted to the PAM approach. With a transmissive liquid crystal SLM there are no moving parts, a low demand on detector dynamic range, and the high tolerances required for interferometry are not present.

Programmable Array Microscopes

Results:

Axial responses: When used to collect conjugate images, measured axial responses to rhodamine thin films indicated a resolution of ~1.0 μm for line patterns having a 1x24 unit cell and ~0.6 μm for the dot lattices having a unit cell of 4x6. A conventional illumination pattern could not discriminate the axial location of the thin fluorescent film. The offsets in these axial responses were characterised in terms of , the ratio of "on" to total modulator elements, and ', the ratio of axial response maximum to minimum. Both patterns had = 0.041, but the line patterns had ' = 0.18 while the dot lattices had ' = 0.38. This implies that a line pattern will show less background than a dot pattern for equivalent , while giving a thicker optical section. In addition, we found that for the same throughput, a random pattern has a lower offset than has a regular pattern of lines or dots.

Observation of biological specimens

The sectioning capability seen with the thin films was also observed in biological specimens (Figure 4). As the spacing of the illumination/detection pattern decreases, the amount of blur from out-of-focus planes increases, as does the amount of cross talk between adjacent apertures in the pattern. Relatively little degradation in image quality is seen between the 20x1 and the 10x1 patterns in the specimen shown. The latter pattern, however, can be scanned twice as fast.

Spectroscopic imaging

Stacks of three-dimensional spectroscopic images can be collected using the spectroscopic system generating a spectroscopic image with 3 spatial dimensions (Figure 5). Similar observations of polytene chromosomes from *Drosophila melanogaster* stained with Alexa594 or Cy3 for the polyhomeotic protein and with YOYO-1 for DNA revealed two spectral signatures, one represented predominantly the fluorescence emission from YOYO-1/DNA and the other from Cy3 or Alexa594. From the four-dimensional data set obtained from observation of *Drosophila melanogaster*, two three-dimensional wavelength slices were extracted and submitted to reconstruction analysis (Figure 6). This procedure allows much of the out of focus blur to be removed giving a better indication of important proteins along the chromosome backbone.

Conclusions

The PAM uniquely combines the power of multiple scanning methods in a single microscope, allowing the results to be compared in a rapid and convenient manner. For any given sample, the selection of an optical sectioning strategy represents a compromise between light throughput, axial resolution, offset rejection, and photobleaching. Deciding which method to use will depend on the sample, the needs of the investigator, and many other experimental variables. The PAM can rapidly adapt its sectioning strategy to best match a particular specimen. In addition, the PAM is a powerful platform on which to develop spectroscopic imaging methods. In both optical sectioning and imaging spectroscopy, the adaptive capacity of the spatial light modulator gives an unparalleled flexibility to the system. Hybrid PAMs combining optical sectioning with emission spectroscopy and lifetime imaging are under development as is the two-sided arrangement shown in Figure 3. ∎
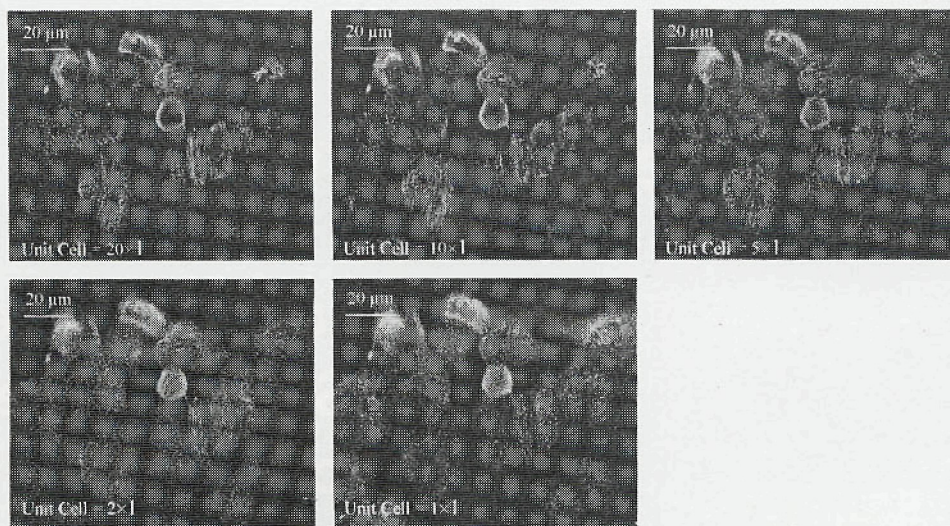
Figure 4. Images of MCF-7 cells stained for tubulin with monoclonal antibody and Oregon green GAMIG, 60x NA 1.4 oil immersion objective.
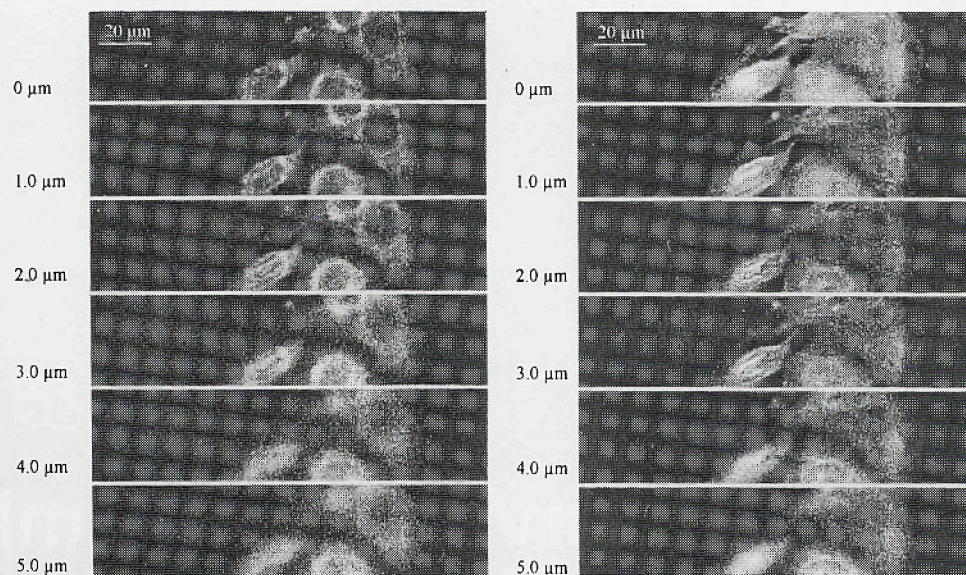
Figure 5 Series of two-dimensional spectral images taken at 1 μm along the z-axis. Each series represents one wavelength slice in the data set. The image series on the left corresponds to approximately 630 nm (MitoTracker). The series on the right corresponds to approximately 525 nm (Oregon Green).
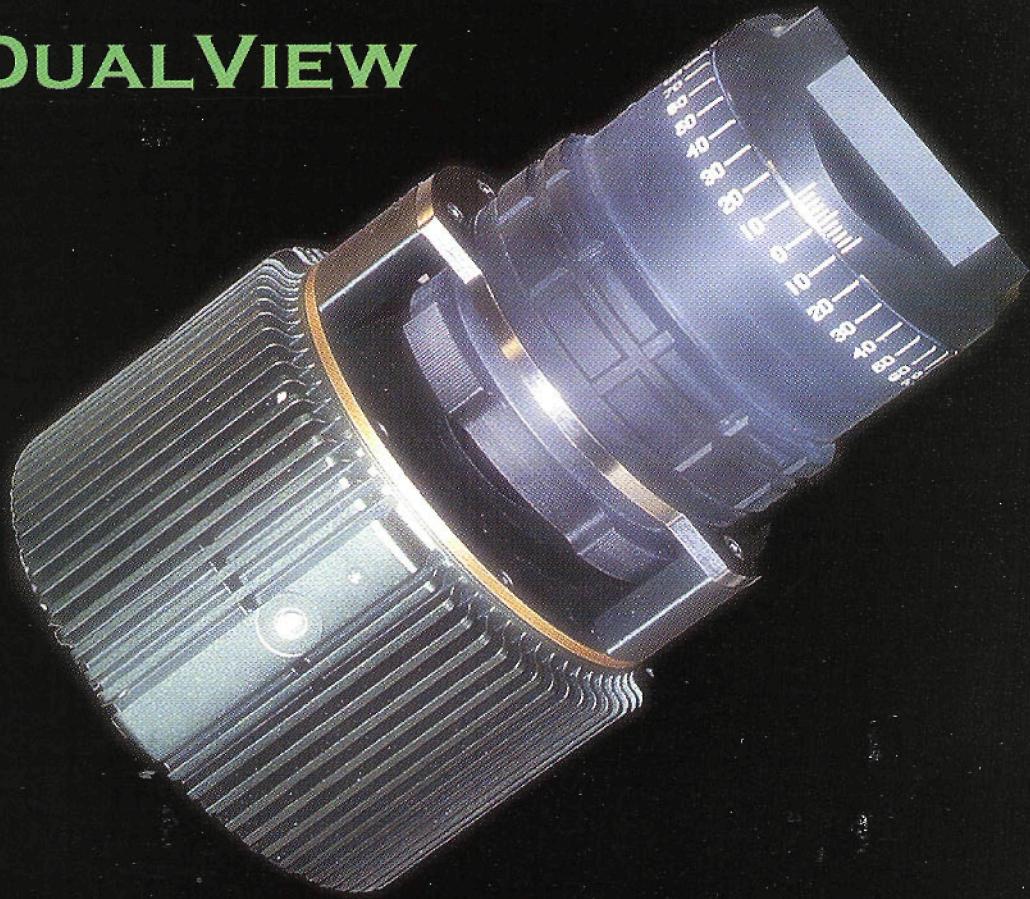
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References:

1) Cha, S. D., P. C. Lin, *et al.* (2000). "Nontranslational three-dimensional profilometry by chromatic confocal microscopy with dynamically configurable micromirror scanning." Applied Optics **39**(16): 2605-2613.

2) Hanley, Q.S., P.J. Verveer, *et al.* (2000). "Three-dimensional spectral imaging by Hadamard transform spectroscopy in a programmable array microscope." Journal of Microscopy-Oxford **197**: 5-14.

3) Hanley, Q. S., P. J. Verveer, *et al.* (1999). "An optical sectioning programmable array microscope implemented with a digital micromirror device." Journal of Microscopy-Oxford **196**: 317-331.

4) Hanley, Q. S., P. J. Verveer, *et al.* (1998). "Optical sectioning fluorescence spectroscopy in a programmable array microscope." Applied Spectroscopy **52**(6): 783-789.

5) Hanley, Q. S., P. J. Verveer, *et al.* (1999). "Spectral imaging in a programmable array microscope by Hadamard transform fluorescence spectroscopy." Applied Spectroscopy **53**(1): 1-10.

6) Juskaitis, R., T. Wilson, *et al.* (1996). "Efficient real-time confocal microscopy with white light sources." Nature **383**: 804-806.

7) Liang, M., R. L. Stehr, *et al.* (1997). "Confocal pattern period in multiple-aperture confocal imaging systems with coherent illumination." Optics Letters **22**(11): 751-753.

8) Malik, Z., R. A. Buckwald, *et al.* (1996). "Fourier transform multipixel spectroscopy for quantitative cytology." Journal of Microscopy **182**: 133-140.

9) Morris, H. R., C. C. Hoyt, *et al.* (1994). "Imaging spectrometers for fluorescence and Raman microscopy: acousto-optic and liquid crystal tunable filters." Applied Spectroscopy **48**(7): 857-865.

10) Puppels, G. J., F. F. M. de Mul, *et al.* (1990). "Studying single living cells and chromosomes by confocal Raman microspectroscopy." Nature **347**(6290): 301-303.

11) Schaeberle, M. D., H. R. Morris, *et al.* (1999). "Raman chemical imaging microscopy." Analytical Chemistry **5**: 175A-181A.

12) Smith, P. J., C. M. Taylor, *et al.* (2000). "Programmable array microscopy with a ferroelectric liquid-crystal spatial light modulator." Applied Optics **39**(16): 2664-2669.

13) Timlin, J. A., A. Carden, *et al.* (1999). "Spatial distribution of phosphate species in mature and newly generated mammalian bone by hyperspectral Raman imaging." Journal of Biomedical Optics **4**: 28-34.

14) Turner, J. F. and P. J. Treado (1996). "Near infrared acousto-optic tunable filter Hadamard transform spectroscopy." Applied Spectroscopy **50**(2): 277-284.

15) Verveer, P. J., Q. S. Hanley, *et al.* (1998). "Theory of confocal fluorescence imaging in the programmable array microscope (PAM)." Journal of Microscopy-Oxford **189**: 192-198.

16) Wilson, T., R. Juskaitis, *et al.* (1996). "Confocal microscopy by aperture correlation." Optics Letters **21**(23): 1879-1881.

17) Youvan, D. C. (1994). "Imaging sequence space." Nature **369**: 79.

Localization of Polyhomeotic on Polytene Chromosomes using Spectroscopic PAM
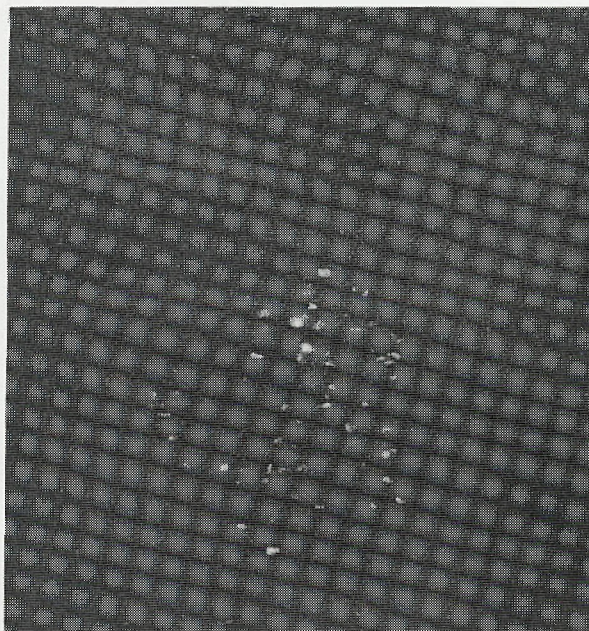
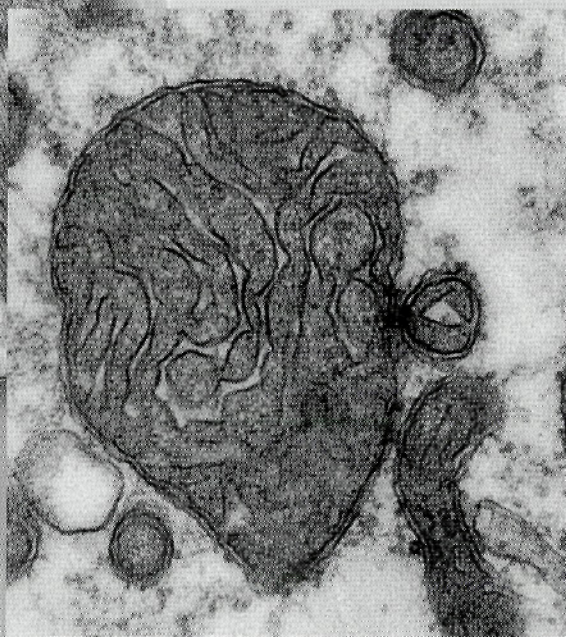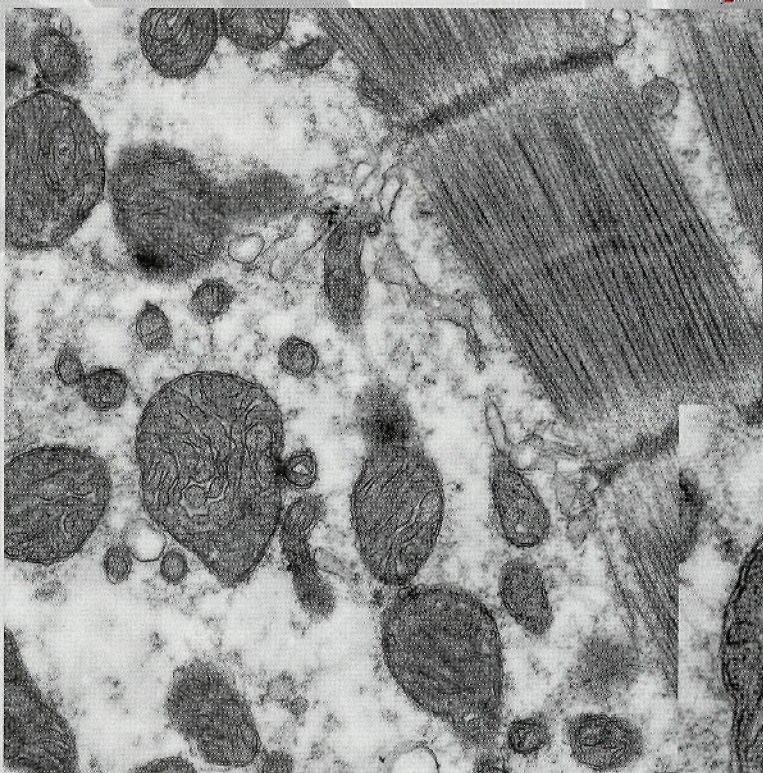**Sequence Length = 511**
100 x NA 1.3 Objective
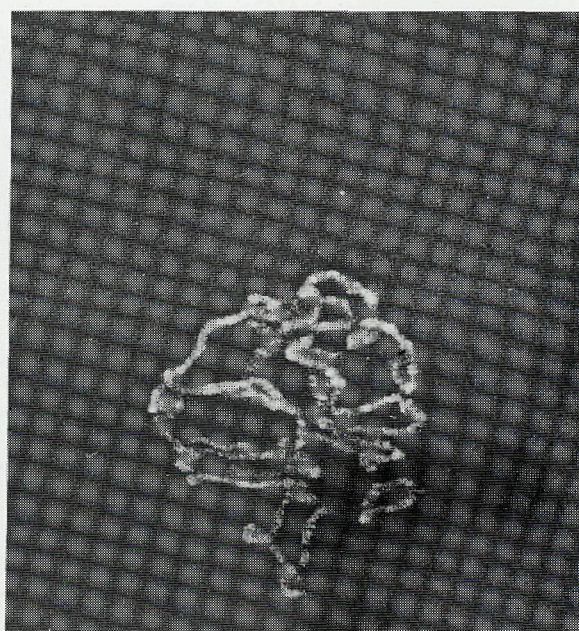
Alexa598 and YOYO-1

Figure 6 Stereo projection representing two spectral segments of a three-dimensional spectroscopic image before (upper panel) and after (lower panel) image reconstruction. This image shows *Drosophila* polytene chromosomes stained for the polyhomeotic protein with Alexa598 and DNA with YOYO-1. Reconstruction is from a stack of 16 spectral images measured at 400 nm intervals in the axial direction. (Data reprinted with permission o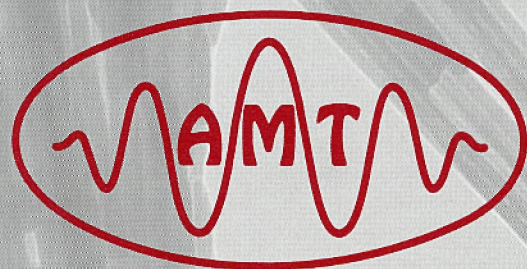f the Journal of Microscopy 197:5-14 (2000)).