High-resolution, multiple optical mode confocal microscope: I. System design, image acquisition and 3D visualization

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ABSTRACT

We have designed and constructed an experimental confocal specimen-scanning microscope which has the capability of producing high resolution 3D images in a variety of optical modes, many of which are not currently available on commercial confocal microscopes. The transmission Nomarski differential interference contrast (DIC) mode is particularly interesting because it can be utilized to image small changes in refractive index within complex biological specimens which are transparent in standard brightfield. The three-color reflection configuration can produce a color 3D image, which means that stained or pigmented objects will be similar in appearance to images obtained from conventional white light microscopes which makes them more recognizable.

1. INTRODUCTION

Confocal microscopes have been under development for over a decade and commercial instruments are now commonly utilized in a variety of applications in the biological and materials sciences as well as for industrial inspection. However, commercial instruments have largely been developed for specific applications and disciplines, which have resulted in the instruments having the capability of high-resolution imaging in only a few of the optical modes common to conventional full-field microscopes. This is a great disadvantage for many users of the instruments, because the confocal images produced are often so different in appearance to the conventional microscope images, that the biologist or materials scientist finds it extremely difficult to identify and interpret what is meaningful information even though the specimen can be viewed as a full 3D reconstruction. Perhaps the most widely used confocal configuration is fluorescence which has a very large number of applications in the biological sciences. This mode produces images that are the most similar in appearance to those of the equivalent conventional microscope (ie. conventional fluorescence) and hence does not present many problems for the typical user. This fact alone may prove to be the major reason for its widespread popularity. However, when moving to the other most common commercial confocal optical mode, reflection brightfield, the problems of locating the areas of interest within the specimen volume and the interpretation of the resulting images are greatly increased. For example, a typical brightfield reflection 2D confocal image will have bright pixels from the in-focus, reflective regions while the remaining areas of the image will be dark. In a true confocal set-up (ie. one where the detector pinhole is of a size to sample only the central 25 per cent of the image Airy disc) the resulting image will often have only a small proportion of bright pixels against a black background.† Hence, each of the 2D images or "optical sections" will have a very different appearance to the conventional microscope view, especially (as in the field of biological microscopy) when the user is accustomed to observing the specimen in a transmission mode where the background is generally light and regions of the specimen appear as various shades of gray. In addition to the identification and interpretation problems encountered, a more fundamental limitation is that commercial confocal microscopes cannot, in general, image phase information in 3D. This is in contrast to conventional biological microscopes in which modes such as Zernike phase contrast and Nomarski differential interference contrast (DIC) are fundamental for the study of living or unstained materials. Furthermore, most confocal systems cannot give an accurate representation of the spectral response of the specimen (ie. relative absorption and reflection of various wavelengths throughout the visible spectrum) which again is in variance to the common use of a white light source and biological stains to provide full-color viewing in a conventional microscope.

A principal reason why commercial confocal systems do not have the range of optical modes typical of a high quality conventional system is that these additional configurations are much more difficult to implement and utilize than the fluorescence mode. A true confocal design uses a small aperture placed at the image plane in front of a photodetector. The correct positioning of this aperture or "pinhole" is crucial if high resolution images are to be obtained. It has been shown in our recent studies of multiple wavelength (ie. color) confocal brightfield or fluorescence imaging[1] that optical effects such as chromatic and spherical aberration can so dominate the performance as to make the images worthless. In addition, in all transmission modes, we have shown that refraction alone can cause the focussed image spot to "wander" both axially and transversely from the position of the fixed detector pinhole[2] which means that almost continual alignment correction is necessary to produce useable images.

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† This is true even if one ignores the problems of coherent speckle which are frequently also present when laser illumination is used and which further compound the problems of identification and interpretation of object features.

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2. A HIGH RESOLUTION, MULTIPLE OPTICAL MODE CONFOCAL MICROSCOPE

In order to produce a much more versatile confocal microscope for 3D visualization of specimens, we have developed a system that has the capability of imaging in both a reflection and a transmission configuration and in addition has the capability of utilizing a range of optical modes such as 3-color (RGB) brightfield, Nomarski DIC and fluorescence. Table 1 provides a summary of all of the optical modes currently under development for high resolution imaging on our confocal microscope and indicates whether they can be used in reflection or transmission.

Optical Mode	Reflection	Transmission
Brightfield (single color)	√	√
Brightfield (3-color RGB)	√	
Nomarski DIC	√	√
Fluorescence	√	
Polarizing	√	√

Table 1: Optical modes of the high resolution, specimen-scanning confocal microscope.

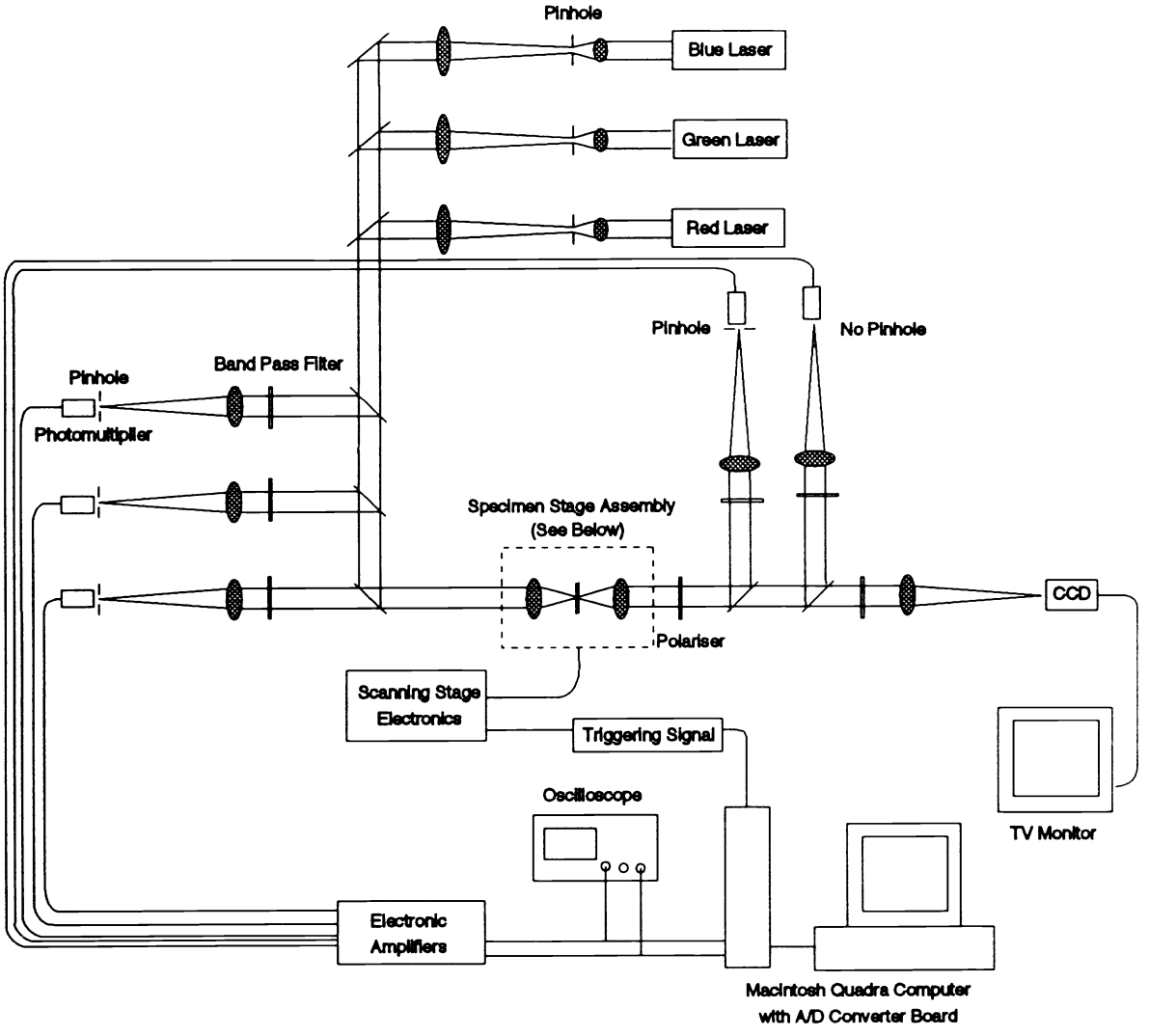
2.1. Design features

The multiple optical mode capability of our experimental microscope has been realized primarily through one fundamental design feature: the illuminating and detected light remains stationary on the optical axis rather than being transversely scanned as it is in most commercial confocal designs. This single feature greatly simplifies the overall optical design and limits all aberrations to an on-axis condition, which means they can be more easily corrected. To produce a 2D image, the specimen (rather than the beam) is mechanically scanned in a raster pattern. The microscope uses very high NA objective lenses (1.4 or 1.3) in order to obtain images having the best possible resolution. Magnification is changed simply by altering the distance over which the specimen is scanned.

2.2. Three-color reflection configuration

The ability to image in color is a powerful tool for a great many applications in biology, geology and materials science and, because of this, white-light illumination has been used in conventional microscopes for centuries. In the twentieth century, due to the design and development of precision multi-element objective lenses ("apochromats"), it has become possible to obtain images that are fully corrected for *lateral* chromatic aberration throughout the entire visible spectrum. If, in addition, the apochromatic objective is corrected for curvature of field ("plan") then its *axial* resolution for visible wavelengths will also be good. However, in order to configure a *confocal* microscope for high resolution color imaging, even when using the most highly corrected planapochromat objectives, it is usually necessary to further correct for chromatic aberration, primarily because the demands for precise axial alignment are greater in confocal microscopes than in the conventional light microscopes for which the objectives are designed.

Because of the constraints imposed by objectives, our experiments with color imaging in a confocal microscope[3] indicate that *axial* chromatic aberration causes severe image degradation if a single multi-wavelength light source and a single detector is used in a confocal system. This is the case even when the most highly corrected objectives are used and despite the on-axis nature of our confocal design which virtually eliminates the lateral chromatic aberration usually present in beam-scanning microscopes. In order to overcome the axial chromatic aberration problem, we have chosen a compromise configuration, one which utilizes three separate illuminating wavelengths (red, green and blue), each with its own individually-aligned illumination and detection path (Fig. 1). This allows three monochromatic images to be acquired simultaneously while the specimen is scanned. Finally, a color image is produced by writing the images to the red, green and blue channels of a display monitor. The three laser wavelengths (HeNe 633nm, frequency-doubled NdYAG 532nm, Argon ion 458nm) provide a reasonable color representation when viewing objects that have fairly broad spectral reflectivity across regions of the visible spectrum, which is the case for many naturally occurring pigments or artificial dyes commonly used in brightfield microscopy. In addition, preliminary results have indicated that our confocal color reflection mode can assist in the location and identification of immunogold particles, 5-10 nm in diameter. These are commonly used probes for labelling biological materials. However, in practice, the gold particles usually require additional enhancement with silver in order to be made large enough for viewing with a conventional light microscope. Our initial studies using the high-resolution color confocal system indicate that sub-wavelength gold particles can be identified by nature of their increased scattering of the green wavelength thus alleviating the need for the silver enhancement process.[3]



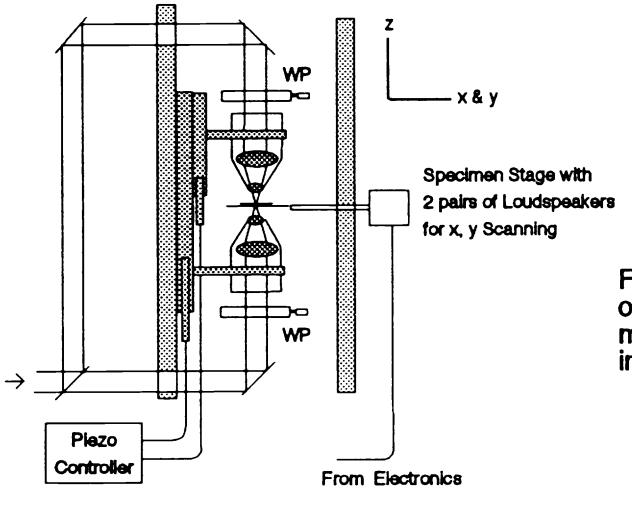


Fig. 1: Schematic layout of the multiple optical mode confocal scanning laser microscope for transmission and reflection imaging.

(WP = Wollaston prism for Nomarski DIC imaging mode)

2.3. Transmission brightfield and Nomarski DIC configurations

Figure 1 also shows the layout of the transmission path of our confocal microscope. In order to obtain high resolution transmission images, several factors must be considered with regard to the overall microscope design and the transmission image formation process. For example, a second high NA objective must be inserted and precisely aligned beneath the object plane and the specimen itself must be mounted between two coverglasses. Preliminary investigations[2] also show that refraction by the specimen will cause the focussed image spot to wander both laterally and axially from the center of the detector pinhole. This factor tends to make high resolution transmission imaging virtually impossible for all but the least complex and thinnest of specimens if a standard fixed pinhole detector arrangement is used. Several mechanisms for tracking the wandering spot and correcting for this problem are currently under investigation in our group.[4] One technique uses a multiple-element array detector which can detect the position of the image spot and then send the information to a tilting mirror system that repositions the spot onto the center of the detector pinhole. Another method utilizes a large area detector such as a high speed CCD array to sample the image Airy disc and explores whether sampling the maximum intensity or the average intensity value at the centroid of the pattern will give the equivalent to a normal confocal detector with a pinhole aperture.

In addition to investigating the problem of the wandering image spot, our studies have addressed a more fundamental issue: that of whether or not confocal transmission imaging modes actually give an improvement in axial resolution over conventional transmission microscopes. In order to conduct comparative studies, our microscope has been configured with two photo-multiplier detectors (PMTs) in the transmission path. The first detector has a pinhole aperture at the image plane (confocal case) and the second utilizes the entire large area of the PMT detector (no pinhole) which simulates a conventional microscope. Using a beam splitter, the light from the transmission objective is split into two image paths in order that a pair of images, one confocal and one conventional, can be acquired simultaneously for comparison purposes.

3. EXPERIMENTAL RESULTS: CONFOCAL TRANSMISSION USING BRIGHTFIELD AND NOMARSKI DIC

In a recent study of thin biological preparations (plant chromosomes) we acquired high resolution confocal images in both transmission brightfield and transmission Nomarski DIC in order to compare the potential of each configuration for imaging lightly stained specimens. For each of these optical modes, two simultaneous through-focus series of images were acquired: one using a fixed pinhole in front of a photomultiplier tube which gave a confocal configuration and another using no pinhole in front of the PMT which, in effect, produced a conventional microscope image. For these initial experiments, simple manual methods for maintaining optical alignment appropriate to confocal transmission were utilized which were based on qualitative assessment of the scanned image and of the Airy disc in the plane of the pinhole. This required realigning the pinhole for each step change in focus which gave a more consistent alignment for all the images in a through-focus stack but was still a compromise with respect to accurate alignment of every pixel in each 2D image.

In both the brightfield and Nomarski DIC experiments, the confocal images showed much improved contrast as well as some subtle improvement in axial resolution over the conventional case. In addition, in this particular chromosome study, the Nomarski DIC images appeared to have much more high spatial frequency information (ie. more fine structure was discernible) than the brightfield images. This is largely due to the well-known capacity of Nomarski DIC to image phase in addition to the amplitude information standardly imaged by a brightfield mode.[5] Thus, slight changes in refractive index in the unstained regions of the preparation are able to be visualized by DIC. Figures 2(a-b) and 3(a-b) show a comparison of confocal versus conventional microscope images of chromosomes using transmission Nomarski DIC, where Fig 3(a-b) represents a plane of focus 1.4 microns below Fig 2(a-b). The tendency of the confocal image to go out of focus more rapidly than the conventional microscope image is apparent. This means that the confocal optical path rejects out of focus information more readily than the conventional case and hence gives an improvement in axial resolution.

A *quantitative* assessment of the resolution of the confocal and conventional transmission images was obtained using Fourier analysis. By taking the Fourier transform of each 2D image slice in a through-focus series, we were able to compare the degree of high spatial frequency information retained in the confocal versus the conventional case. Figures 2(c-d) and 3(c-d) are 2D Fourier transforms (FTs) which represent the spatial frequency content of each image. The center of each FT corresponds to the lowest spatial frequency with progressively higher spatial frequencies radiating outward vertically and horizontally. The lateral asymmetry of the FTs is due to the optical characteristics of DIC which produces a shading gradient ("bas relief") effect in one of the transverse directions.

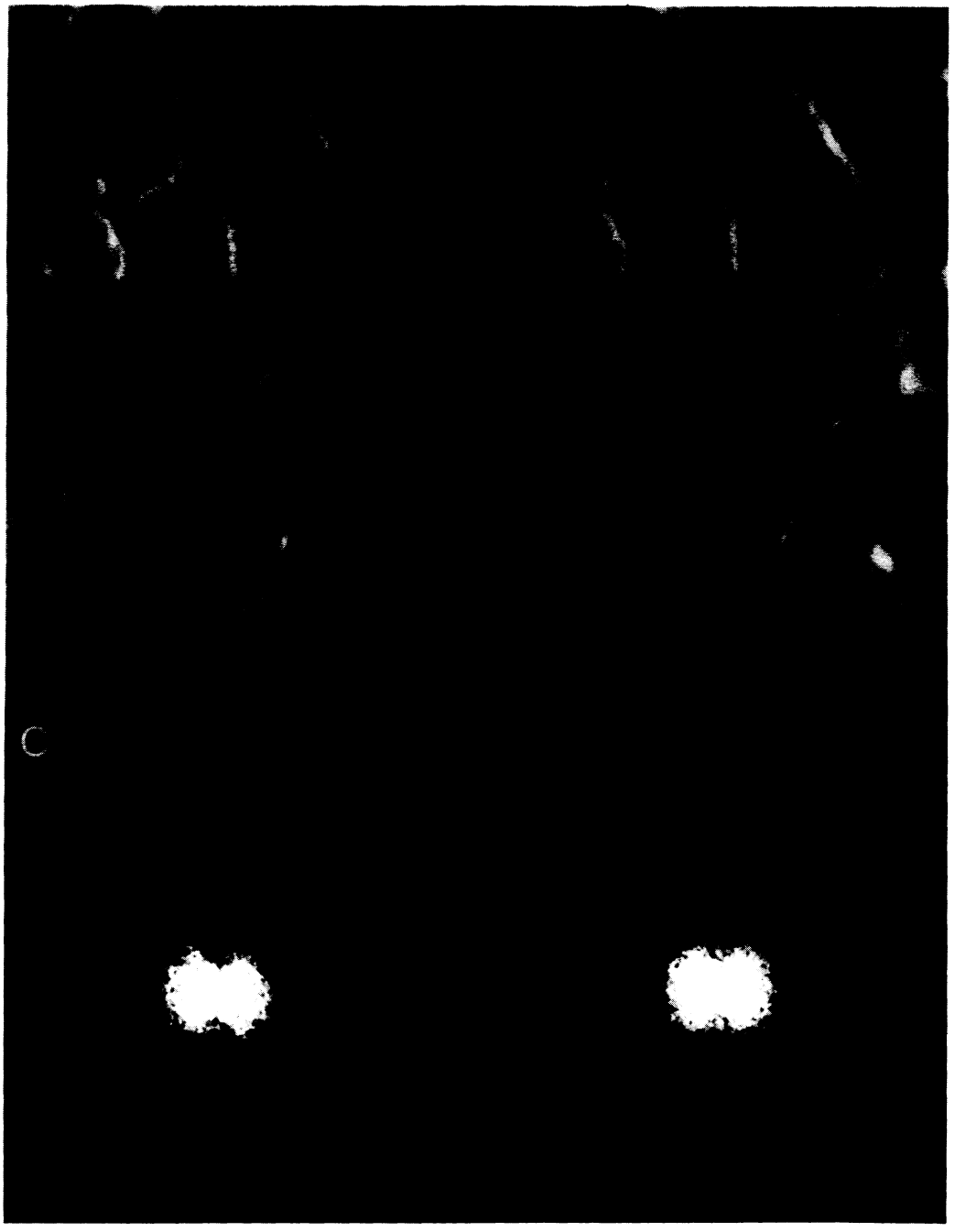


Fig. 2. Focussed transmission DIC images of metaphase orchid root tip chromosomes acquired simultaneously (a) with a 10 micron detector pinhole (confocal) and (b) with no detector pinhole (conventional microscope). The Fourier transform for each image is shown in (c) and (d) respectively. The arrows indicate a feature which has gone out ot focus in the pinhole case, but is still visible in the no pinhole case.

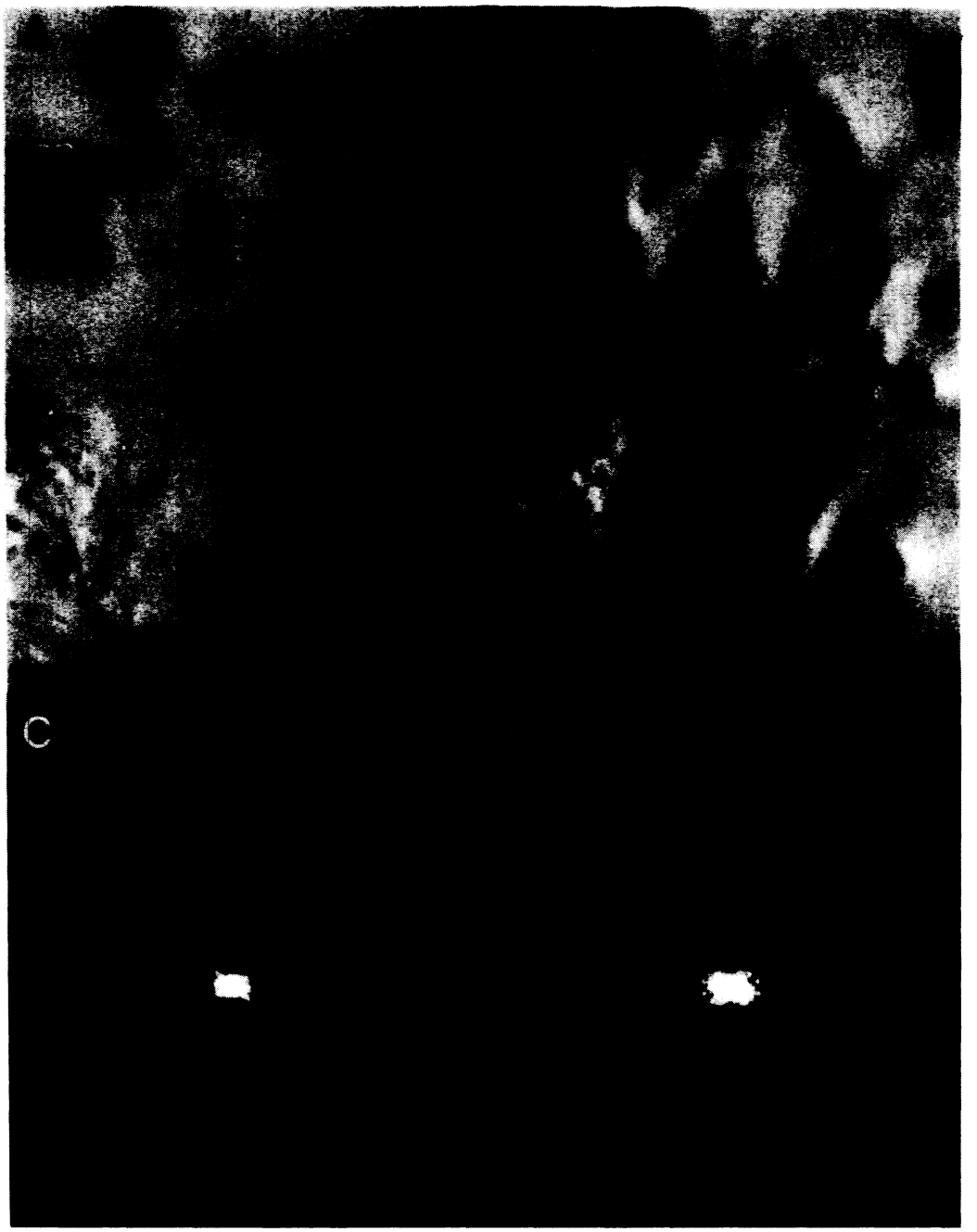


Fig. 3. Images of the same chromosomes as Fig. 2, but a. a plane of focus 1.4 microns below Fig. 2. (a) detector with pinhole and (b) detector with no pinhole. The respective 2D Fourier transforms are shown in (c) and (d). The pinhole case (a) shows more defocus of features than the conventional (no pinhole) case which demonstrates the ability of the confocal detector to reject out of focus information more readily. This is also clearly visible as a reduction in the percentage of high spatial frequencies in the confocal Fourier transform (c).

4. CONCLUSION

Use of confocal scanning laser microscopy (CSLM) in transmission presents new problems in optical alignment. In reflection or fluorescence CSLM the beam deflection introduced by refractive index boundaries within the specimen is cancelled out on the return path, so that the focussed spot falls consistently upon the fixed pinhole detector. This does not occur in transmission, causing the spot to "wander" across the pinhole as it passes through inhomogenous media in the specimen. In a recent study of thin specimens (plant chromosomes) we have acquired high resolution confocal images in both transmission brightfield and transmission Nomarski differential interference contrast (DIC) using a fixed pinhole. In the process, methods for maintaining optical alignment appropriate to confocal transmission have been found using qualitative assessment of the scanned image and of the Airy disc at the plane of the pinhole. Comparisons have been made between the confocal (detector with pinhole) and conventional (detector without pinhole) microscope configurations by recording two images simultaneously. The confocal case showed much improved contrast, and preliminary results indicate improved axial resolution for the confocal case. Analysis of the Fourier transforms of the data also allowed a quantitative assessment of transmission imaging properties.

Transmission CSLM also presents new problems for the 3-D visualization of confocal data. As predicted by theory, confocal transmission brightfield images acquired on our microscope do not exhibit total removal of out of focus regions from the image, in marked contrast to the reflection brightfield and fluorescence modes of confocal microscopy. In order to render such data in 3-D, image processing must be performed to remove the out of focus regions of the image. In addition, transmission Nomarski DIC produces 2-D sections with a *bas relief* appearance, which also requires image processing in order to present this information in 3-D. In both cases, the image processing techniques need to take account of the optical characteristics of the respective imaging modes of the confocal microscope.

Using the multiple optical mode confocal system, other optical defects such as defocus and spherical aberration can also be readily monitored and we are furthering our investigations into methods for interactively detecting and correcting these types of aberrations. The ultimate goal of the project will be to produce a user friendly confocal microscope which is capable of 3-D imaging in all of the optical modes commonly found in conventional microscopes, and will include the capacity to produce simultaneous images which are a combination of reflection and transmission information.

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