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ADVANCES IN VISUALIZATION OF MITOSIS IN VIVO[1]

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ABSTRACT A new form of light microscopy, single-sideband edge-enhancement microscopy, has been devised. Microscopes of this form extend our ability to monitor mitosis in individual living cells. Using illumination that is not absorbed by the object, the image is intensity modulated as a function of optical path difference through the specimen or as a function of rate of change of optical path through the specimen or as a combination of both. Thus, the image can resemble a phase contrast image with much reduced halo and improved resolution, a differential interference contrast image, or intermediate combinations of both. The optical path differences to which the microscope responds are both those due to isotropic differences in refractive index and those due to birefringence. In the latter case the effective direction of the electric vector of the light probing the specimen is selected after the light has passed the objective and may be changed without moving the specimen.

INTRODUCTION

Visualization has two principal meanings: one refers to the process of forming a mental image or concept; the other is the process of making somthing visible. Even within the limitations imposed by the wavelength of visible light, observation of living cells in the act of mitosis contributes in an important way to our conception of the mechanisms of mitosis. The extent to which these events can be rendered visible conditions our interpretation of electron microcope analysis and in vitro biochemistry of mitosis.

The transparency of living cell structures, particularly the mitotic apparatus, necessitates use of special contrast enhancing microscopes such as phase contrast, polarizing, interference and differential interference contrast (DIC)

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microscopes. Each has contributed both to our ability to see and to understand mitosis.

SINGLE-SIDEBAND MICROSCOPY

Small transparent objects produce invisible phase modulated images in brightfield microscopy because the light diffracted by the object arrives at the image plane 1/4 wave out of phase with the direct light of the illuminating beam. This was suggested to Zernike (1) by the phenomenon illustrated in figure 1, in which a piece of transparent diffraction grating replica sprinkled with carbon particles is shown as it appears above, below and near best focus. Away from best focus the diffracted light is shifted ahead of or behind phase quadrature and a fuzzy intensity modulated image of the grating is formed by interference with the direct light. At best focus, the diffracted light is in phase

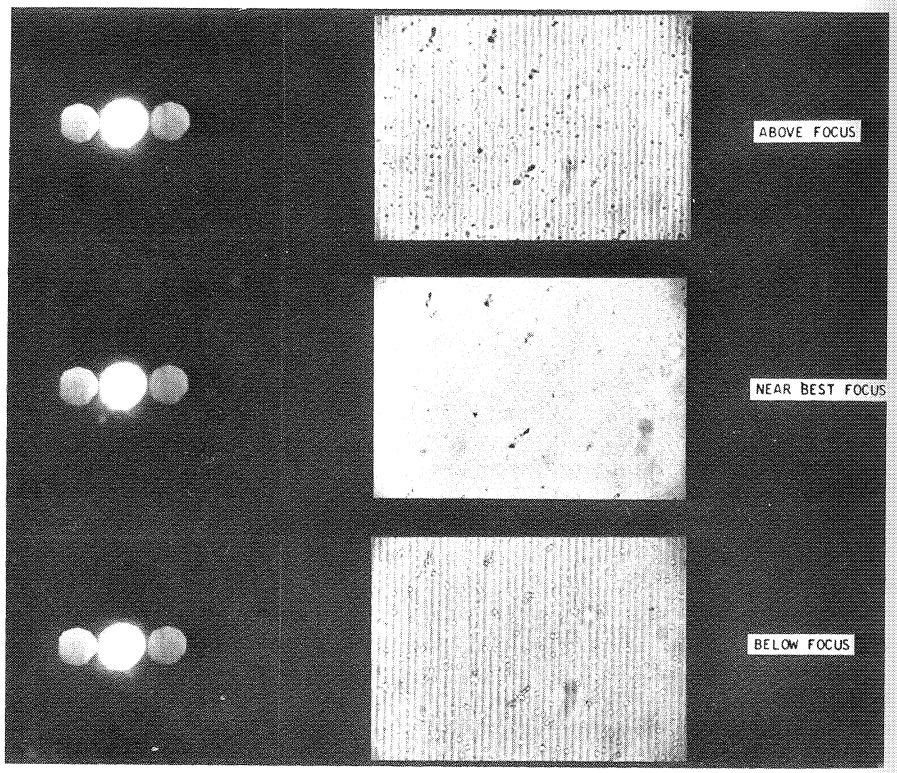

Figure 1. Objective aperture (left) and image for a phase object viewed above, near, and below best focus.

quadrature and can neither add to nor subtract from the background intensity produced by the direct light. The phase contrast microscope converts the phase modulated image into an intensity modulated image by changing the optical path length of the direct light by 1/4 wave to bring it either into phase or 1/2 wave out of phase with the diffracted light at the plane of best focus.

Figure 2 shows that while the sum of both sidebands of light diffracted by the transparent object is in phase quadrature with the direct light (which we may now regard as the carrier wave), the light in each sideband includes components that are more or less than 1/4 wave out of phase, as is shown by the fact that they are able, in the absence of the other sideband, to interfere with the direct light to produce intensity modulated images at the plane of best focus. For the sum of both sidebands to be shifted by 1/4 wave relative to the direct light requires that the diffracted

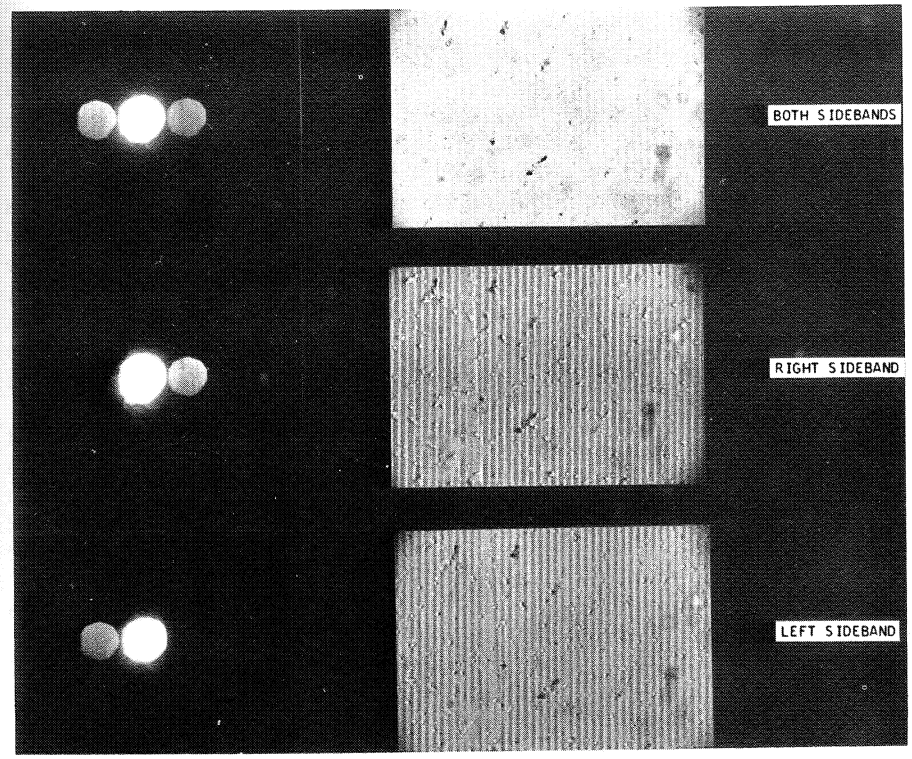

Figure 2. Objective aperture (left) and focused image using both, right, and left sidebands. Object as in figure 1.

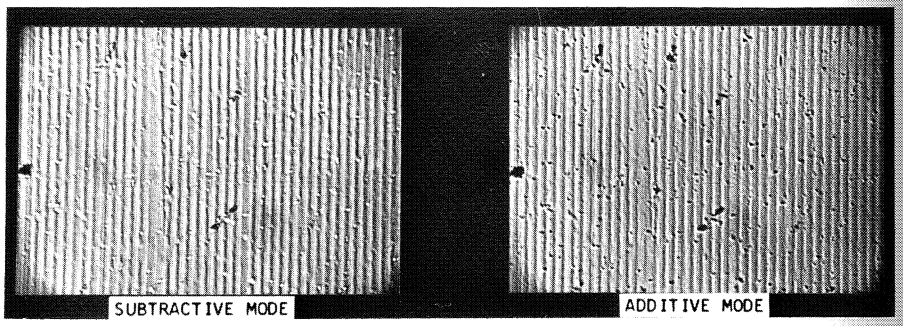

Figure 3. Phase object imaged by microscope of figure 4.

light components that deviate from quadrature in one sideband must be shifted by a complementary amount in the opposite sense in the other sideband. That this is so is verified by the fact that the image formed using one sideband shows transparent objects in reversed contrast relative to the image formed using the other sideband, while the use of both sidebands produces no image of the transparent structure. Though not illustrated, the deletion of both sidebands arising from the grating, by specifically blocking these diffracted orders with opaque dots, eliminated the image of the grating regardless of focus, but left the carbon particles' images little changed.

In figure 3 we see the same test object as imaged by a microscope (figure 4) designed to controllably favor one sideband over the other and to regulate the amplitude of the carrier admitted to the image plane. In the additive mode, diffracted light exclusively from one sideband is combined at full amplitude with the much attenuated carrier (including the coincident components from the lower spatial frequencies of both sidebands).

In the subtractive mode the amplitude of the carrier and its superimposed low frequency sideband components is, in addition to being attenuated, shifted 180° in phase relative to the unaltered sideband light. The resulting reversal of image contrast confirms the diffraction-interference character of the image formation process.

Hoffman and Gross (2) prefer to account for the phenomenon shown in figure 2 by what amounts to a geometrical optical argument that invokes refraction of zero order light out of the primary image. At a scale where the dimensions of the object are not large compared to the wavelength of the illuminating light, this argument is not compatible with physical optics. Images formed as in figure 2 are understandable in concordance with Zernike's additions to Abbe's (3) theory of microscope image formation if one

considers the fact that transparent objects, as exemplified by blazed diffraction gratings, do not necessarily diffract energy equally into both sidebands.

Lord Rayleigh, cited in Strong (4), predicted this in an article in the 1880 edition of the Encyclopaedia Brittanica. The images formed by Modulation Contrast® microscopes, particularly the oblique illumination form offered commercially, must be regarded as single-sideband images.

THE SINGLE-SIDEBAND EDGE-ENHANCEMENT (SSEE) MICROSCOPE (5)

The SSEE microscope (figure 4) consists of:

1. A high intensity light source, in the present case a 100 watt Hg concentrated arc lamp (Osram HBO 100 or equivalent).

2. A well corrected lamp collector lens to provide uniform illumination that utilizes efficiently the high intrinsic brightness of this source. The lens presently used is Condenser assembly #11800-855 from the American Optical Co., Scientific Instrument Division,Buffalo, NY 14215. It is used in their A.O. Project-O-Chart opthalmological projector and identifying it as such will help expedite orders. Insertion of a 1-1/4" by 1/16" silicone rubber O-ring between the lens retaining cap and the last lens element retains the lens elements at their correct spacing while allowing for differential expansion during warm-up. This corrects a dismaying tendency for the first lens element to crack during warm-up.

3. The field diaphragm. Not shown are the heat absorbing filters (Corning #4602) and wavelength selecting interference filter, usually a Baird Atomic high transmission type B2 for 546nm green light but for cells susceptible to damage by green light , a similar filter selecting the 577 and 579nm yellow lines is used.

4 & 5. The condenser iris and half stop. The condenser iris is set to match the effective na of the condenser to that of the objective used. The half stop is generally set to occlude 1/2 of the condenser aperture, or slightly more, making the entrance pupil of the microscope a near semicircle whose radius is proportional to the objective na.

6. The condenser, which should be well corrected for spherical aberration and capable of matching the na of the objective used. In the present system a Bausch and Lomb 1.4 na achromatic-aplanatic condenser is used. It has been generally satisfactory but a longer working distance would be desirable for working with cells within culture chambers.

7. The specimen support. The microscope must have a rotatable stage.

8. For the objective any brightfield objective may be used

subject only to tube length and coverglass limitations. The new plan-apochromats, from several sources, provide spectacular results.

9. An insertable rotatable 1/2 wave birefringent retardation plate. This is used to post select the effective direction of electric vector vibration in the specimen when examining birefringent objects.

10. The relay lens, which images the back focal plane of the objective on the carrier attenuation filters.

11. The first image of the specimen (not directly accessed in this microscope).

12. A rotatable polarizer used in conjunction with the next three components to control the amplitude of the carrier and its phase displacement relative to the selected sideband.

13. The carrier attenuating filter (CAF), described below, is positioned so that its division boundary concides with the image of the chordal edge of the condenser entrance pupil and is confocal with it. Depending on the type of filter used, adjustment of the polarizer or analyzer or both allows selective attenuation of the carrier (also known as illuminating beam, direct light and zero order) relative to the selected sideband (a.k.a. scattered light, indirect light and diffracted orders). Because of the specific geometry of the condenser entrance pupil and the carrier attenuation filter, all the components of one sideband, along all specimen azimuths, are either excluded from the objective or attenuated along with the carrier, while much of the other sideband is transmitted undimmed. Hence image formation is predominantly by interference between the attenuated carrier and the freely transmitted sideband. The portions of the selected sideband which pass through the carrier side of the filter, and are attenuated, are those bearing low to intermediate spatial frequencies to the image. Therefore, in images formed by this microscope, the higher spatial frequency components are enhanced in contrast.

The elements of the CAF are mounted between antireflection coated glass cover plates. Several types have been tested. They are:

Type A filters which use birefringent elements of either 1/4 or 1/2 wave retarding materials for the two halves. One may have its slow axis oriented in any azimuth relative to the division; but the other must have its slow axis at 45° to the first. These birefringent CAF's are aligned for use by orienting the polarizer with its electric vector transmission direction parallel to the slow axis of the carrier side of the filter. Carrier extinction occurs with the analyzer crossed to the polarizer.

Type B filters which use polarizing elements oriented with their transmission directions at 90° to each other. These

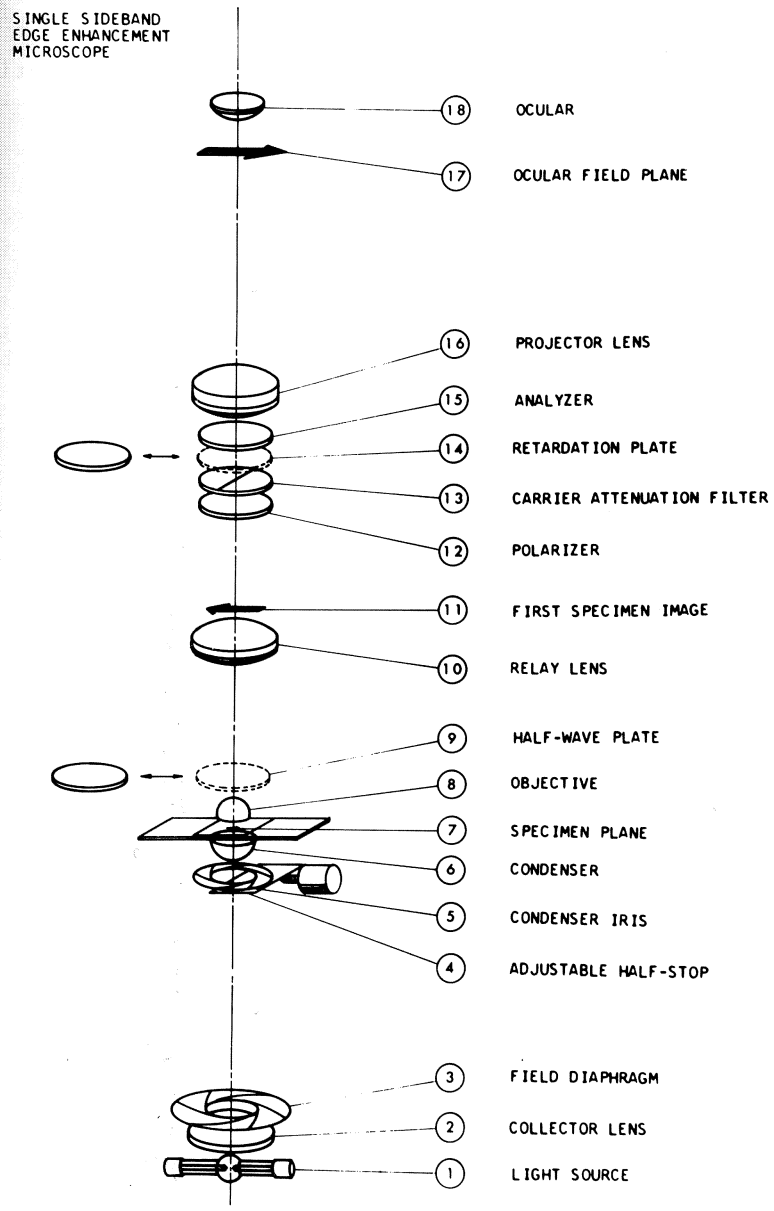

Figure 4. Schematic diagram of the SSEE microscope.

filters are used by aligning the polarizer at a small angle to the transmission direction of the sideband side of the filter.

The analyzer is then set parallel to the polarizer for additive mode (sideband added to the carrier at the image), or at a similar angle to the other side of the filter direction for the subtractive mode (sideband subtracted from the carrier).

Type C filters which are made of a single circular sheet of stretched polyvinyl alchol (PVA) stained on the carrier side with iodine. This makes the carrier half of the filter a dichroic polarizer whose transmission direction parallels the slow axis direction of the birefringent unstained sideband half of the filter. In use the polarizer or analyzer is set at a small angle to the fast axis of the sideband side of the filter and the analyzer or polarizer is rotated a suitable angle away from carrier extinction in either the additive or subtractive direction.

Type D filters which are type C filters with an unstained circle of PVA laminated to the filter, with its slow axis crossed to that of the filter, to compensate the birefringence of the sideband half to make it effectively isotropic. Used with the compensating PVA sheet toward the polarizer, this filter provides pure single-sideband amplitude contrast, as does the type B filter, unless a retardation plate or compensator follows the filter. With the unstained PVA sheet toward the analyzer, type D filters act similarly to type C filters.

For any of the carrier attenuating filters described above changing the analyzer setting from one side of extinction to the other reverses the phase of the carrier relative to the transmitted sideband. Hence for opposite rotation of the analyzer (and/or polarizer) from carrier extinction, transparent specimen detail will generally appear in reversed contrast. Highly refractile objects presenting large optical path differences are the exceptions.

14. The insertable retardation plate or compensator is used with the carrier attenuating filters which introduce no phase shift between carrier and transmitted sideband to provide Zernike type phase contrast where desirable.

15. Rotatable analyzer.

16. The projection lens relays the specimen image to the ocular field plane.

Parts 10 through 16 of the author's pilot model SSEE microscope consisted of the intermediate tube of a Nikon model #77000 interference-phase attachment with its 1/4 wave plate removed and the phase plate slider replaced with a carrier attenuating filter and, as needed, a retardation plate or compensator.

17. The ocular field plane.

18. Ocular(s) - any that are compatible with the color correction and tube length of the objective.

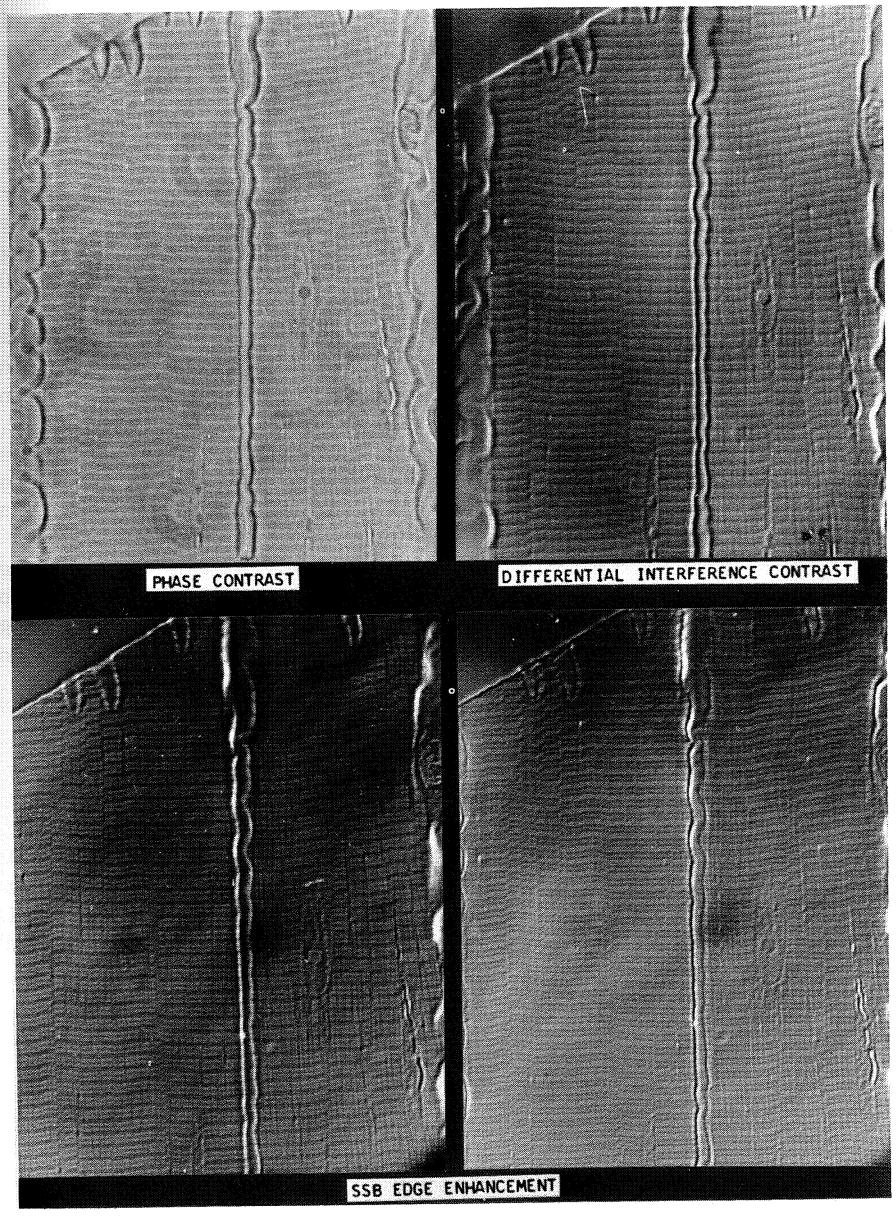

Figure 5. Comparison of phase contrast, DIC, SSEE additive mode (right) and SSEE subtractive mode.

RESULTS

Inoue has devised a standardizable test object intended for use in polarization microscopy but useful also as a phase object. This consists of sections of araldite embeded, glutaraldehyde fixed frog sartorius muscle cut at appropriate thickness on an ultramicrotome. The sarcomere repeat interval has been determined, by diffraction of 632.8nm HeNe laser light, to be 2.2nm. The test slide used here has sections cut at 360nm and at 180nm mounted in Euparal and was graciously provided by Dr. Inoue. Figure 5 shows a comparison, using this test slide, between phase contrast using a Zeiss 40x 1.0 na HI phase apochromat,Zeiss - Nomarski DIC with the standard 40x 0.65 na planachromat and SSEE using an Olympus 40x 0.95 na Plan Apo objective. These micrographs of the 360nm section clearly show the superiority of SSEE over phase contrast in resolution, depth discrimination and, at this thickness, contrast. A type C filter was used and the phase shift this provides makes the increased optical path, through the a-bands and z-lines relative to the i-bands, easier to determine than with DIC, independent of picture orientation.

The test object for figure 6 is the head of a mature living sperm of the cave cricket. These sperm show no discernible internal structure when examined by non-polarizing optical means, appearing as smooth refractile rods. SSEE is compared with Nikon

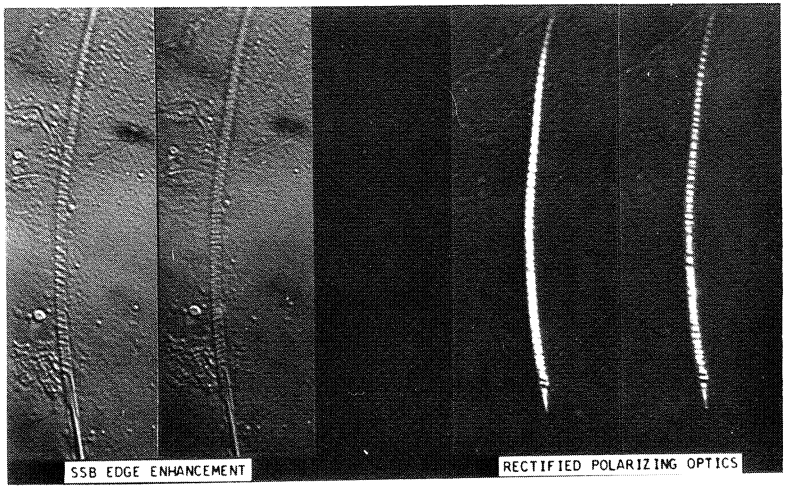

Figure 6. Living cave cricket sperm.

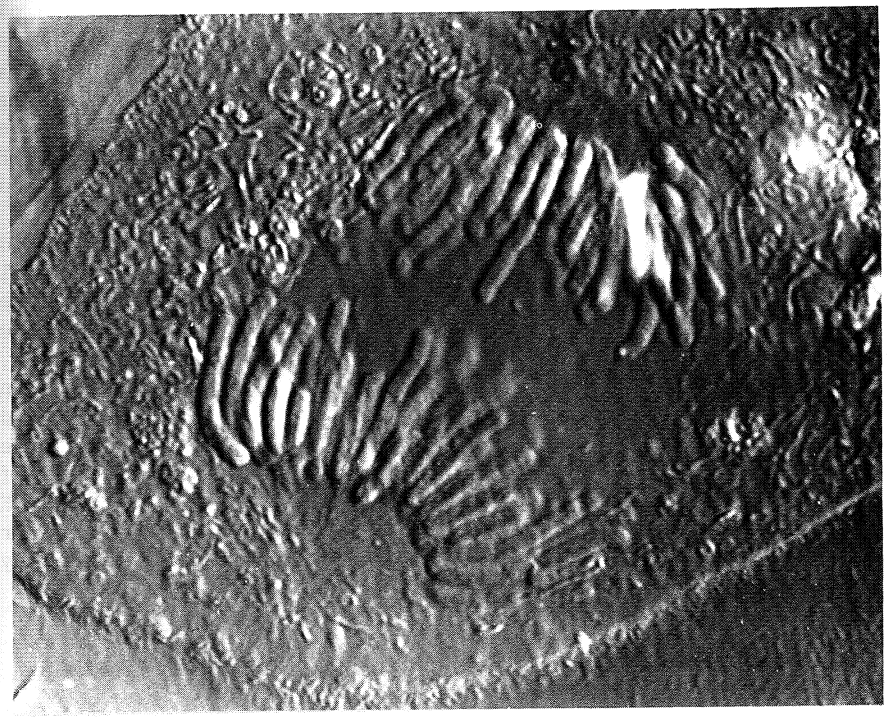

Figure 7. Newt lung epithelial cell in culture.

rectified polarizing optics using the same 100x 1.25 na rectified objective and 8 mm, 1.15 na condenser for each.

The final example, in figure 7, is a cultured newt lung epithelial cell undergoing mitosis in an intact Rose chamber complete with cellophane substrate. The objective used is a Zeiss 63x 1.4 na HI planapochromat. This picture could not be made with DIC or polarizing microscopy.

DISCUSSION AND CONCLUSIONS

The optical path differences to which the SSEE microscope responds are both those due to isotropic differences in refractive index and those due to birefringence. In the latter case the effective direction of the electric vector of the light probing the specimen is selected after the light has passed the objective and may be changed without moving the specimen. In common with both phase contrast and DIC systems, contrast is enhanced for higher spatial frequency components of the specimen image, thus yeilding improved definition at edges. Unlike most phase contrast systems and in common with

all DIC systems image contrast in SSEE images is a function of specimen orientation. However, unlike DIC images, the directional dependence of image contrast in SSEE images varies with spatial frequency, with the highest spatial frequencies the least dependent on orientation. Consequently, the directionality of image contrast is less obtrusive with SSEE than with DIC. Nevertheless, a rotatable stage should be used with either system.

In the form described, the single-sideband edge-enhancement microscope does not require special objectives or condenser turrets, does not require expensive crystalline components and does not require that the objective and condenser operate between crossed polars. As a result, such microscopes should be economical and versatile in application. Culture chambers, perfusion chambers and temperature and/or pressure control chambers, which can be difficult to use with polarizing or DIC microscopes because of chamber birefringence, are readily accessible to SSEE microscopy. Therefore, observation of mitotic events under many different experimental conditions can be facilitated by use of single-sideband edge-enhancement microscopy.

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COMPARATIVE ANALYSIS OF STABILITY CHARACTERISTICS OF HEXYLENE GLYCOL AND DMSO/GLYCEROL ISOLATED MITOTIC APPARATUS[1]

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ABSTRACT We have shown that mitotic apparatus isolated with dimethylsulfoxide/glycerol (DMSO/glycerol) support chromosome movement when they are injected into enucleate frog eggs, as judged by normal cleavage of these eggs. In order to ascertain properties of the DMSO/glycerol mitotic apparatus that are essential for sustaining chromosome movement we have investigated the stability and chemical composition of the mitotic apparatus under various conditions of isolation. In addition we have compared the stability of mitotic apparatus isolated using various methods (DMSO/glycerol and hexylene glycol). Birefringence of hexylene glycol mitotic apparatus are dependent upon the pH of the isolation medium, - the higher the pH the lower the birefringence. The stability of the hexylene glycol mitotic apparatus is also dependent upon the pH of isolation medium, - the lower the pH the slower the rate of birefringence decay. DMSO/glycerol isolated mitotic apparatus have more stable birefringence than hexylene glycol mitotic apparatus. The birefringence decay of the DMSO/glycerol isolates were about 1000 times slower than the isolates prepared with hexylene glycol. We are currently studying the proteins found in the mitotic apparatus under various isolation and storage conditions, using polyacrylamide gel electrophoresis, and we are investigating the movements of sea urchin zygote chromosomes after mitotic apparatus are injected into frog eggs.

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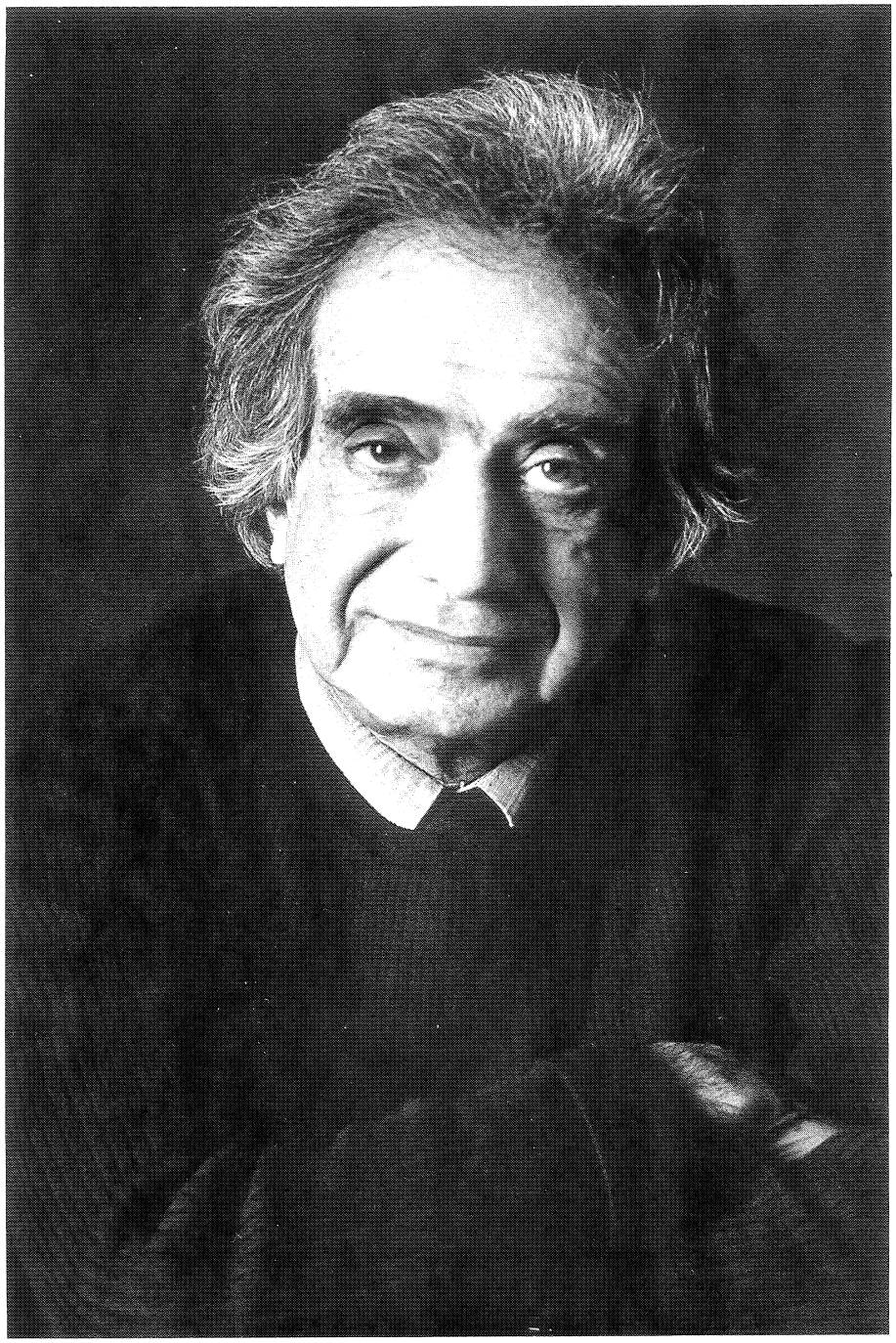

Photograph by Ruth Mayerson Gilbert

DANIEL MAZIA

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CELL REPRODUCTION: IN HONOR OF DANIEL MAZIA

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