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PHASE-RANDOMIZED LASER ILLUMINATION FOR MICROSCOPY

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SUMMARY

A simple apparatus is described that phase randomizes the output of a continuous argon ion laser, so that it may be used as a source of high intensity, monochromatic light for microscopy. The phase-randomizing device can be used with any laser, polarized or unpolarized, and of any desired power output and wavelength, including dye lasers for spectral studies. The randomizing system can be adapted to any light microscope and any optical system including bright-field, phase-contrast, Nomarski differential-interference, dark-field, and split-beam interference systems such as the Jamin–Lebedeff System. It can be used for a variety of photometric and photographic studies. The 514-nm wavelength appears to be relatively harmless to a variety of cells.

INTRODUCTION

Light-microscopical approaches to many biological problems are hampered by light sources of insufficient intensity. This is especially true when the light is required to be monochromatic and/or polarized. In such applications, photographic exposures of the order of 10–100 s are often required at high magnifications. Lasers offer a possible solution to this problem because of their high intensity.

Although lasers have been applied to a variety of biological problems, they have not found widespread use as illumination sources for microscopy. This is attributable in part to the coherence of laser light. Light scattered by dust or minor imperfections in lens components, slide, coverslip, etc., produces a complex interference pattern which causes pronounced deterioration of image quality. A previous attempt to 'phase randomize' laser light by spinning an etched plastic disk in the beam (LaFountain, Muckenthaler & Allen, 1968) succeeded in ridding the image of major anomalies, but some diffraction artifacts (halos around spherical objects, etc.) still remained.

We describe here a simple apparatus for phase randomizing the output of a continuous laser. It produces a significant improvement in image quality and is applicable to a wide range of microscopical systems.

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MATERIALS AND METHODS

The complete illumination system is demonstrated in Fig. 1A, B.

A Zeiss photomicroscope was used in this study because of its convenience for both 35-mm still and 16-mm ciné photomicrography. However, the phase randomizer is adaptable to any microscope. To minimize vibrations, the microscope is bolted to a heavy block which is fastened to a wall-mounted bench. The microscope is secured to the block after first aligning it to a wall-mounted camera (Arriflex, M-16).

The laser used was a 5-W, continuous, Argon ion laser (Model 165, Spectra-Physics, Inc., Mountain View, California). This instrument offers four output wavelengths in the visible spectrum: 476·5, 488·0, 496·5 and 514·5 nm. The 514·5 line has been used routinely because of its high output power and the apparent lack of deleterious effects of irradiation with this wavelength on the living cells employed in our studies. The laser was mounted on a screw-driven positioning device, permitting both vertical and lateral displacement for alignment to the microscope.

The alignment of the laser is accomplished in 3 stages. (1) Coarse alignment is carried out by directing the unexpanded laser beam through the microscope with the objective, condenser, substage, etc., removed. (2) The alignment is then refined after objective and condenser have been replaced. (3) Finally, a beam expander is added to the laser and centred to the optical train. Care must be exercised when aligning the unexpanded beam because of its high-energy density. Standard film polarizers, such as those found in the substage and upper Nomarski Wollaston mounts, and absorption filters which are not rated for high energy inputs will be irreparably damaged by the unexpanded beam unless it has been considerably attenuated.

The procedure for aligning the laser should begin by centring the stage to the optical axis of the microscope so that a cross-hair or other marker on the stage remains in the centre of the field when the stage is rotated. Coarse alignment (step 1 above) is easily carried out using the Zeiss Photomicroscope I because it has 2 field apertures that lie in the optical axis of the microscope. Using the lateral and vertical traverse adjustments of the laser mount, the beam is centred in these 2 apertures and on a cross-hair in the ocular or camera viewing device.

For step 2, a tungsten source is added to the microscope, the objective and condenser are replaced and centred to the stage, the source is removed, and the laser beam is aligned quickly, precisely and safely in the following way.

The image of the unexpanded beam in the field diaphragm plane is projected through one of the eyepieces on to a sheet of white cardboard. Then, using a Bertrand lens in place of one ocular, the back focal plane of the objective is similarly projected. The laser is adjusted until the beam is precisely centred in the field plane (projected through the ocular) when either field stop is closed down. In the back focal plane of the objective (projected through the Bertrand lens), the diffraction rings from the closed field stop should be symmetrical and centred within the iris diaphragm when the laser beam is properly adjusted. This method provides precise alignment according to wave-optical criteria and avoids the hazards to vision of direct viewing of the laser light.

Alignment is completed (step 3) by adding a beam expander and collimator (Spectra-Physics. Inc., Models 331 and 334) to the laser and centring the expanded beam to the microscope with the controls on these two devices.

The phase-randomizing system consists of a relay lens system designed to produce Köhler illumination, a rotating glass wedge, and a ground glass diffuser. The 2 lenses (Fig. 1B) are placed a distance apart equal to the sum of their focal lengths. Lens 1 (simple, plano-convex, 3·2 cm diam., $f = 5{\cdot}2$ cm) focuses the expanded beam on a plane conjugate with the back focal plane of the objective. Lens 2 (simple, plano-convex, 4·3 cm diam., $f = 3$ cm) recollimates the beam, filling the field diaphragm. Since the light is monochromatic, simple lenses are preferred in order to miminize light losses. It is desirable to have the lenses and wedges anti-reflexion coated for maximum transmission of the 514-nm wavelength.

A circular, optical glass wedge of 4 cm diameter (see Results) is placed in a mount on the inner race of a rotatable motor bearing which is driven at 2500 rev/min by a 50-W (1/15 Hp) synchronous motor. The wedge is placed in the collimated beam close to lens 1 to minimize light losses. The wedge, mount, and motor must be mounted independently of the bench

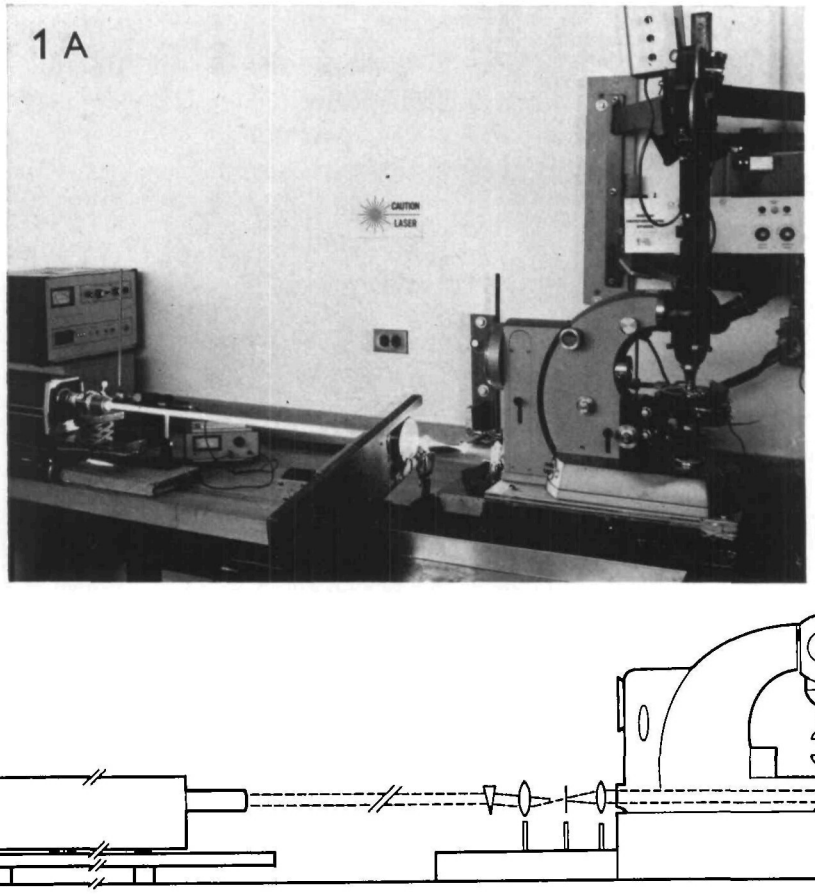

Fig. 1. A, photograph of laser plus phase-randomizing system taken with laser light. B, diagram showing components of phase-randomizing system placed between the laser and microscope. From left to right components are: optical glass wedge, lens 1, ground glass diffuser and lens 2.

supporting the microscope in order to prevent vibrations from being transmitted from the phase randomizer to the optical system. A ground glass diffuser is then placed just in front of the focal point of lens 1.

RESULTS AND DISCUSSION

The need to phase randomize laser light for light microscopy is plainly evident in Fig. 2A, a Nomarski differential interference micrograph of human buccal epithelium taken using coherent laser light. The image of the cell is almost totally obscured by optical noise generated by a multiplicity of interference phenomena.

The simplest way to reduce the coherence of a laser beam is to pass it through a stationary ground glass. However, sufficient coherence remains to make the ground glass itself visible in the image plane. If the ground glass, or better still an etched plastic disk, is spun at high speed (LaFountain *et al.* 1968), the image quality is

greatly improved. However, some artifacts persist. In Fig. 2B, the field is not evenly bright and a series of asymmetric surface fringes extend partly around the cell.

Both the field inhomogeneity and surface fringes are eliminated by the rotating wedge system described here (Fig. 2C). Furthermore, light losses from the system are

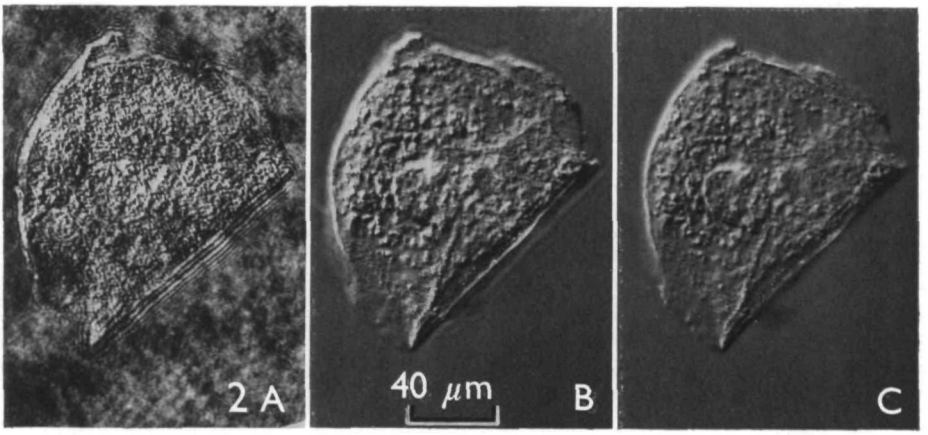

Fig. 2. Nomarski differential-interference photomicrographs of a human oral epithelium cell taken under the following illuminating conditions. A, non-randomized laser source. The image of the cell is almost totally obscured by optical noise. B, laser source randomized with spinning disk (see text). Note surface fringes which extend around the cell due to incomplete randomization, C, laser source randomized with rotating-wedge system. Field is homogeneous and surface fringes are absent.

Table 1. *Optimal wedge angle for a variety of microscope objectives*

Objective	Optical wedge angle, °
16× planachromat, N.A. = 0·35 (Zeiss)	3
40× planachromat (dry), N.A. = 0·65 (Zeiss)	4
40× planachromat (oil), N.A. = 0·85 (Zeiss)	4
100× planachromat (oil), N.A. = 1·25 (Zeiss)	6

less than one-half those of the spinning disk method. The spinning optical wedge rotates the collimated beam through the outer region of lens 1. This lens brings the light into focus in the form of an annulus which is imaged by lens 2 in the back focal plane of the objective. The ground-glass diffuser scatters the light, completely filling the front focal plane of the condenser. The amount of light scattered out of the annulus depends on the position of the diffuser relative to the focal point of lens 1. The overall effect of the illumination system is a homogeneously and fully illuminated aperture and field plane, and a minimum of image artifacts due to excessive coherence.

The wedge angle has an important effect on both the image quality and light losses. Light losses are minimal if the diameter of the annulus (viewed in the back focal plane with the ground-glass filter removed) corresponds to approximately 75% of the

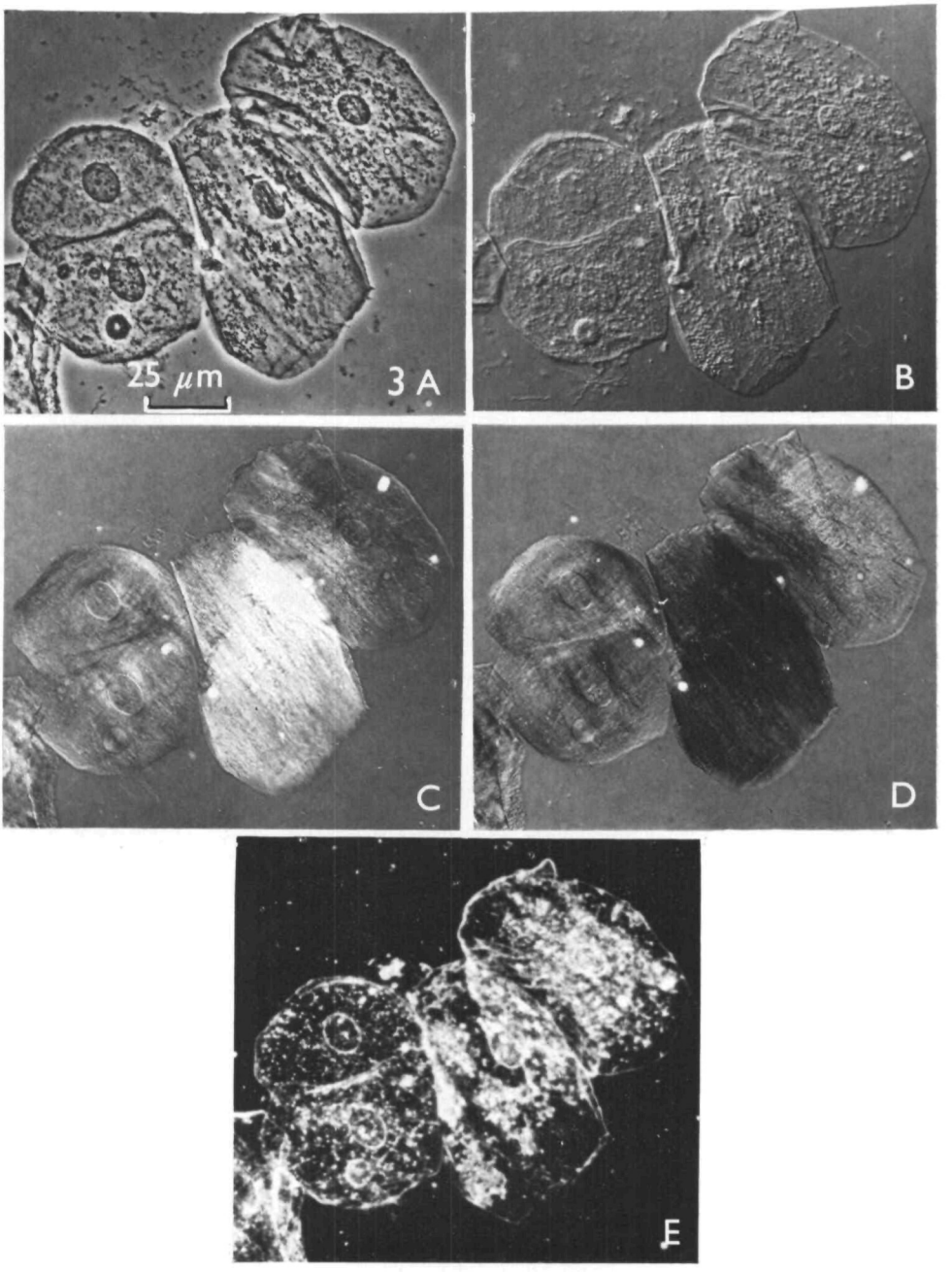

Fig. 3. Photomicrographs of human oral epithelial cells taken with laser light source using a variety of optical systems. A, Zeiss positive phase-contrast (40 × dry objective, N.A. = 0·65). B, Zeiss nomarski differential-interference contrast (40 × oil-immersion objective, N.A. = 0·85). C, Zeiss strain-free polarized light (40 × oil-immersion objective, N.A. = 0·85). D, as in C, but opposite bias compensation. E, Zeiss dark-field (16 × objective, N.A. = 0·32).

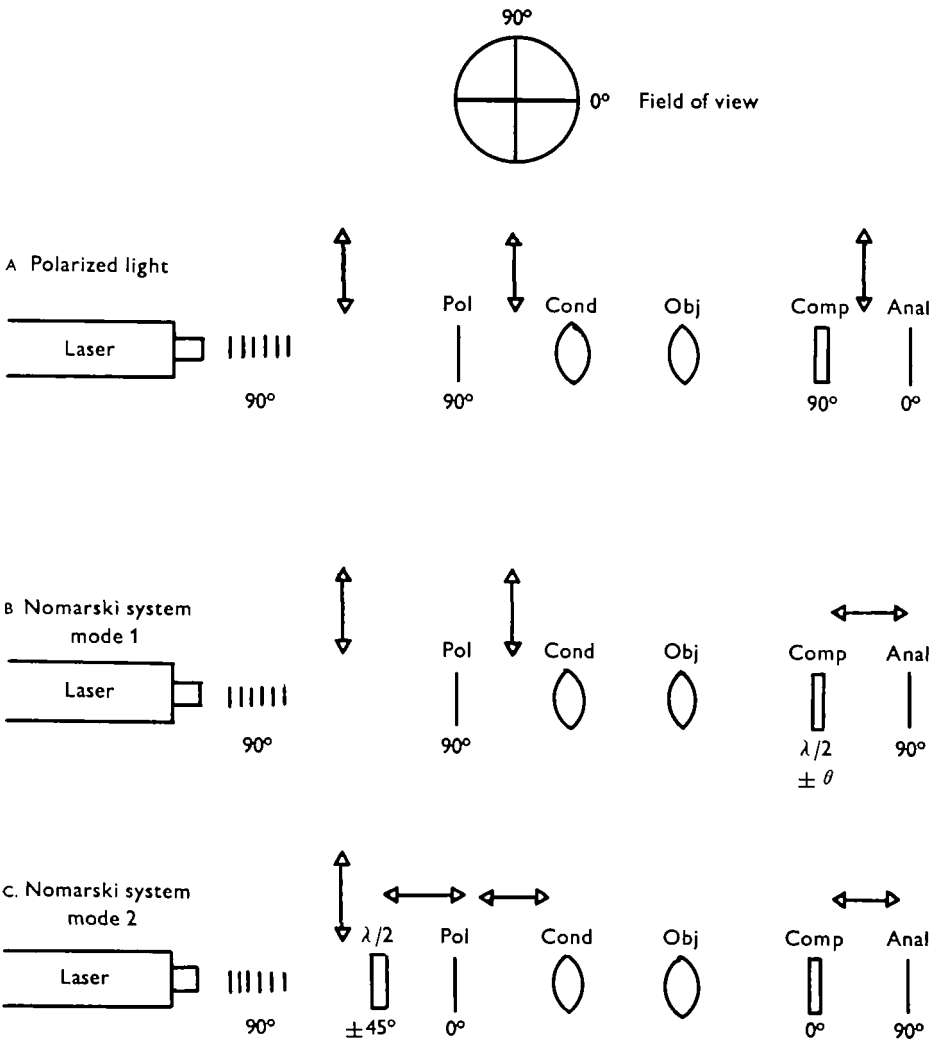

Fig. 4. Diagram illustrating the orientation of components in polarizing and Nomarski differential interference systems. All orientations are as indicated relative to the field of view. Double arrows indicate state of polarization under extinction conditions for light as it passes through optical train. A, polarized light. Anal, analyser; Comp, compensator; Cond, condenser; Obj, objective; Pol, polarizer. B, Nomarski differential-interference mode 1 (see text). Comp = Nomarski upper Wollaston prism set for $\lambda/2$ retardation on zero-order fringe. Other designations as in A. C, Nomarski differential-interference mode 2 (see text). $\lambda/2$ plate is used to rotate beam 90°. Other designations as in A.

diameter of the back aperture of the objective used. The optimal constructed wedge angles for a variety of objective lenses are given in Table 1.

The phase-randomization device has been tested with a variety of optical systems commonly used in cell biology, and with each it has provided exceptional results. These systems include phase-contrast, Nomarski differential-interference, polarized-

light, dark-field and Jamin–Lebedeff interference optics. The quality of images obtainable is exemplified in Fig. 3.

Phase-randomized laser illumination is especially valuable when used with optics requiring polarized light. Conventional light sources lose from 50 to 80% of their light intensity merely upon polarization, and lose considerably more once they are rendered 'monochromatic' by a narrow-band interference filter. With the argon ion laser, these light losses do not occur, since the source is already monochromatic and polarized.

For use with polarized-light optics, the substage polarizer is oriented parallel to the polarization direction of the laser for maximal light transmission (Fig. 4A). This corresponds to the north–south direction when viewing the microscope field. Less than 45% of the light is lost on repolarization. The analyser is crossed to the axis of the polarizer and compensation is achieved using de Sénarmont compensation with a fixed $\lambda/4$ retardation plate designed for 514-nm wavelength and rotatable analyser, or with variable azimuth compensators of the Köhler type calibrated at 514 nm (Bennett, 1950).

There are 2 modes of operation for Nomarski differential-interference contrast (Fig. 4B, C). In the first mode, the polarizer is oriented parallel to the polarization plane of the laser. The analyser in the upper Wollaston mount is fixed by the manufacturer at an azimuth which is also parallel to that of the laser. However, one can achieve extinction even when the polars are parallel by setting the Nomarski compensator to produce uniform half-wave retardation on the first-order red fringe. This rotates the beam 90° so that it is crossed to the analyser. Rotation of the compensator about this half-wave setting allows for the usual amount of bias compensation.

The second mode is preferred as it results in higher extinction factors and better image quality. In this mode, the polarizer is oriented in the usual manner, crossed to the axis of the fixed analyser. An inexpensive plastic half-wave plate (Polaroid Corp.) is placed on the field diaphragm mount with its crystalline axes oriented 45° to the plane of polarization of the laser. The net effect is the same as the first mode with the half-wave plate rotating the plane of polarization of the laser beam so that light is maximally passed by the polarizer. Compensation is achieved in the usual manner. By orienting the half-wave plate at angles other than 45°, the intensity can be varied and kept within safe limits for viewing objects under study.

For photographic purposes, the high intensity of the laser allows short exposures with high-resolution, fine-grain films. This applies to 35-mm (Kodak Panatomic X, Kodak Microfile, and Adox KB14) as well as 16-mm ciné films (Kodak Fine Grain Recordak). With some weakly birefringent objects used in polarized light studies, as much as 200-fold reductions in exposure times have been obtained when compared to a conventional HBO-200 mercury arc light source filtered with a narrow band interference filter for 546 nm.

If photographic or photometric data are to be collected at high frequency, one must take into consideration the fact that exposures that are short compared to the speed of the rotation of the wedge will restore the optical noise that phase randomization seeks to eliminate. One complete revolution of the wedge can be considered as a minimum acceptable exposure time or data-gathering interval.

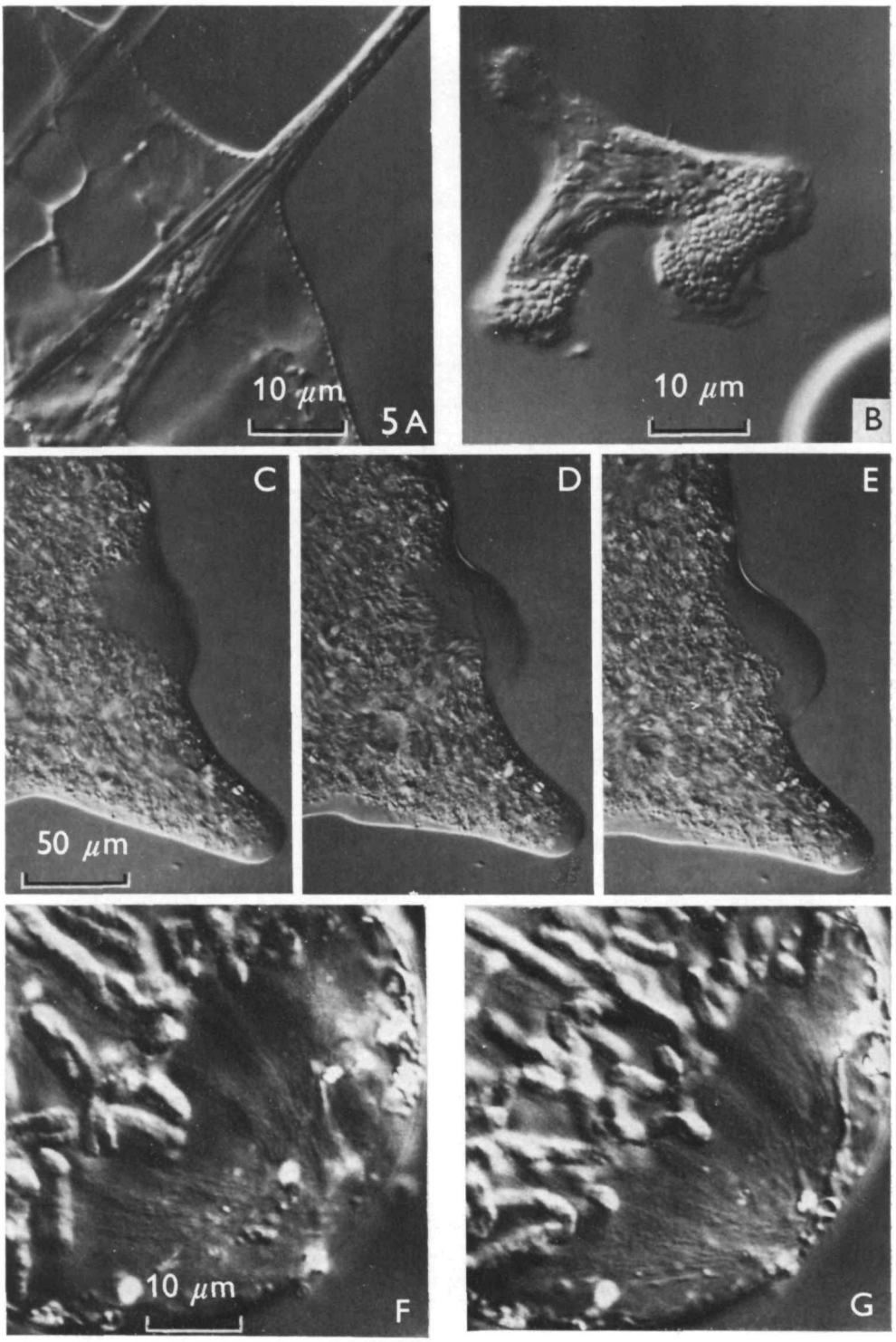

Fig. 5. Nomarski differential photomicrographs of a variety of cell types used to investigate the effects of laser illumination systems on living processes. A, *Echinosphaerium nucleofilum*. Demonstrates bifurcation of axoneme on entering cell body. Note apposition of cell membrane at juncture. B, eosinophylic leucocyte from newt, *Taricha granulosa*, showing hyaline region of advancing pseudopods. C–E, *Chaos carolinensis*, showing initial stages of pseudopod formation. F, G, endosperm cells from *Haemanthus katherinae*, showing kinetochore fibres in metaphase and anaphase, respectively.

Once phase randomized, the laser may be used as a conventional source of high intensity and monochromatic light. We have tested the system with a number of living cells to determine whether the laser source might cause deleterious effects on living processes. In addition to the dividing crane fly spermatocytes used in LaFountain's studies (LaFountain, 1972*a*, *b*), these cells include living amphibian blood granulocytes, *Nitella* internodal cells, the giant amoeba, *Chaos carolinensis* and smaller species of amoebae, dividing endosperm of *Haemanthus katherinae*, and the heliozoan, *Echinosphaerium nucleofilum*. No discernible injury or differences in behaviour have been detected in comparison with conventional sources. The photomicrographs of several cell types in Fig. 5 demonstrate the quality of images achievable with the illumination system on a variety of cell types.

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