References and Notes

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Structure and Organization of the Living Mitotic Spindle of Haemanthus Endosperm

Abstract. *New details of mitotic spindle structures in the endosperm of* Haemanthus katherinae *(Bak) have been demonstrated by differential interference microscopy. Spindle fibers are clearly seen in the living spindle extending from the kinetochores to the polar region. Individual spindle fibers consist of a bundle of smaller filaments which diverge slightly from the kinetochore and intermingle with filaments from other spindle fibers as they approach the polar region. The degree of intermingling increases during metaphase and anaphase. The chromosomes stop moving when the spindle fibers are still 5 to 10 microns long; then the fibers disappear. These observations explain some aspects of spindle movements which were difficult to reconcile with earlier concepts of spindle organization.*

Although spindle fibers were depicted by cytologists of the last century (*1*) from studies on fixed and stained material, these fibers were probably not seen in normal living cells until Cooper (*2*) observed them in the division of the blastomeres of a mite, with the bright-field microscope. The early work of Inoué (*3*) established that the spindle contains birefringent, longitudinal bands that have been assumed to be equivalent to spindle fibers seen in fixed cells. Since that time, polarized light microscopy has been the method of choice for study of the living mitotic spindle.

Some years ago, Bajer and Molè-Bajer (*4*) introduced a promising new material for the study of mitosis, the endosperm of *Haemanthus katherinae* (Bak). Nuclei of these cells (or syncytia in the earlier development of the endosperm) are large and give rise to 27 chromosomes ($N = 9$), the largest of which are about 100 μ long in late prophase. In flattened cells, the mitotic spindle is often nearly 70 μ long and the equatorial plate 100 μ wide (*5*). In a series of studies, the motions of chromosomes and other particles in and on the spindle have been recorded by frame-by-frame analysis of phase-contrast time-lapse films (*6*). *Haemanthus* endosperm is probably the best plant material now available for studies of the details of chromosome movement during mitosis.

Haemanthus endosperm is also an ideal material for the study of spindle structure, organization, and function, but spindle fibers are seldom seen in living cells except under polarized light, and least of all in cells that go on to divide. One of us has reported (*7*) that of several hundred cells observed with phase-contrast optics, in only about 5 percent that divided in a perfectly normal way were spindle fibers clearly observed.

The invisibility of the spindle fibers of most dividing cells with sensitive phase-contrast or interference microscopes has been interpreted in the past (*8*) as indicative of insufficient difference in optical path because of a good refractive-index match between the fibers (if they exist as seen in fixed and stained material) and their matrix. It occurred to one of us (R.D.A.) that another explanation might be the inherent insensitivity of phase-contrast microscopes under the conditions in which spindle fibers are looked for. (Spindle fibers may be considered as thin, linear structures lying side by side in the plane of focus so that each is partially obscured by its neighbors' halos; in addition, particulate and fibrillar structures above and below their plane of focus contribute phase disturbances that degrade the image.)

We therefore undertook a joint preliminary examination of dividing *Haemanthus* cells in the Nomarski differential interference contrast system (*9*) [hereafter referred to as the Nomarski system (*10*)].

The detail that can be observed in the cover photograph and in Figs. 1–3, which show the same cell at later stages, readily attests to the elegance of the Nomarski system for the investigation of the living mitotic spindle. Comparison of Fig. 1*a* (phase contrast) and 1*b* (Nomarski system) immediately shows a wealth of detail in Fig. 1*b* that is either missing from the phase-contrast photograph or partly obscured by phase disturbances above and below the plane of focus. Most noteworthy are the readily distinguished fibrillar elements in the spindle and the substructure visible in the chromosomes.

The image obtained with the Nomarski system and other differential interference contrast microscopes deserves brief explanation, since this class of instruments may be unfamiliar to many readers. The "optical shadow-casting effect" in the image is not caused by any kind of anaxial illumination, nor is it to be interpreted as a representation of a third dimension. Instead, the distribution of brightness in the image of, for example, a spindle fiber corresponds to the derivative of the brightness distribution that would be observed in an ordinary interference microscope with bias compensation. Thus, instead of the contrast being all negative or all positive, depending on the bias compensation, as in normal interference contrast (*11*), the contrast appears sharpest where the rate of change in optical path is greatest; it is positive on one side of the object and negative on the other. The effect, therefore, is as of a "shadow"—almost as if side lighting were used—but the direction from which the shadow is cast depends upon the analyzer setting and on whether the object is phase-advancing or phase-retarding relative to its immediate surround. Since the optical system makes use of a very small lateral optical shearing, the system has a fixed directionality. Therefore, objects lacking spherical symmetry must be rotated on the stage to reveal different features of interest. An important characteristic of the Nomarski system is relative freedom from phase disturbances from structures above and below the plane of focus. Thus, at high working apertures, the field is quite shallow. The system uses polarized light to effect its beam separation, and therefore birefringence is visible if sufficiently strong. However, the spindle fibers observed in the present study were resolved as refracting rather than as birefringent bodies.

Several new findings about the structure and organization of the *Haemanthus* spindle have emerged from only these preliminary observations and photographs taken with the Nomarski system.

1) Spindle fibers are clearly visible as refractile bodies in nearly every dividing cell when the Nomarski system is properly adjusted to emphasize them.

2) Spindle fibers can be traced all the way from the kinetochore of the chromosome to the polar region. The fiber can be seen to consist of a bundle of numerous filaments which diverge slightly from the kinetochore and intermingle with filaments originating from other kinetochores. Thus the polar region can be said to be "loosely organized." In flattened cells, the individual bundles maintain their individuality for a greater distance than in unflattened cells as they converge toward the polar region.

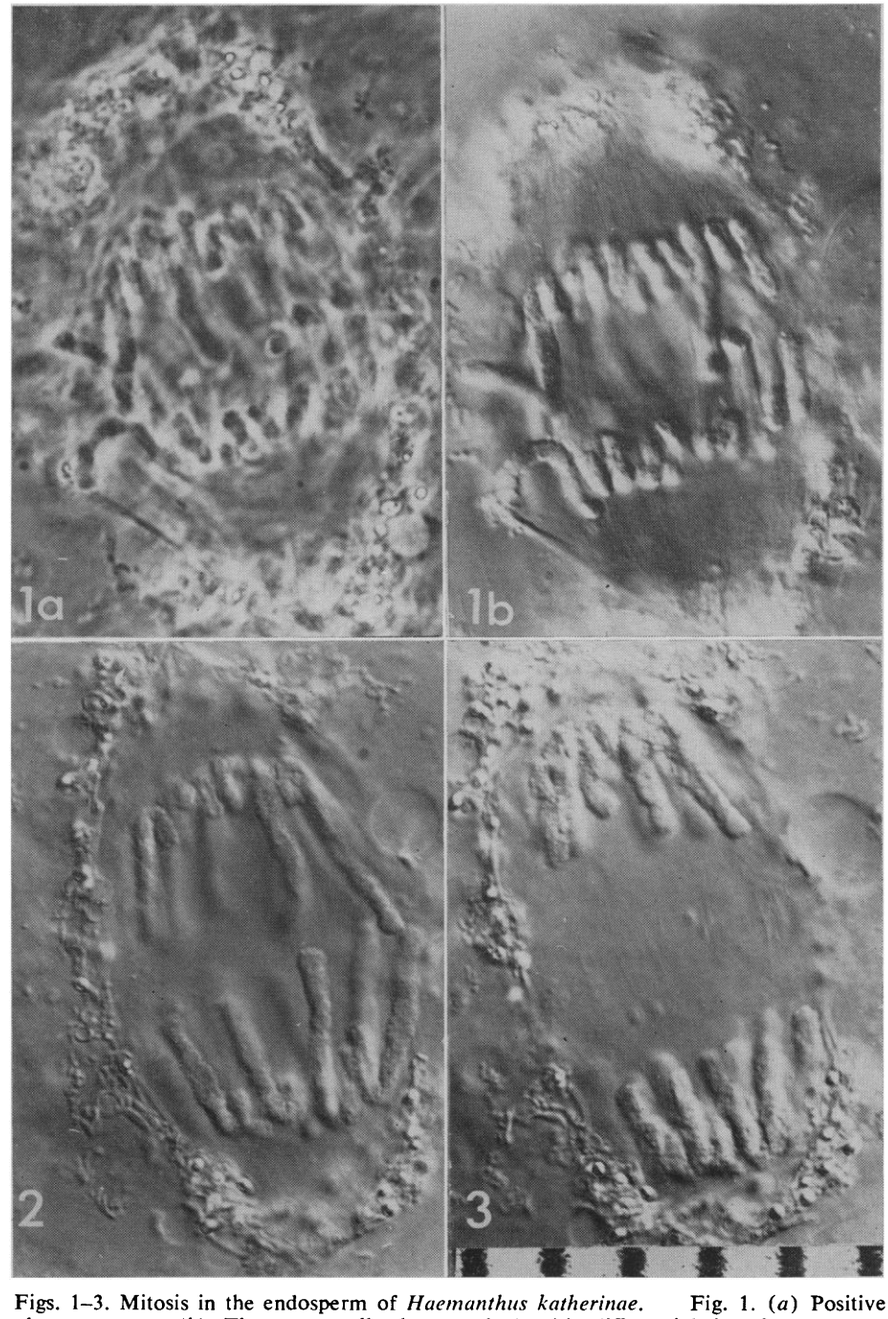
3) Close inspection of motion pictures and of enlargements clearly shows not only that each chromosomal spindle fiber [using the terminology of Schrader (*1*)] consists of a bundle of refractile elements, but also that the single elements have a beaded appearance, the significance of which is still obscure. In some cases the bundles are twisted, in other cases not. It is clear that it is this entire structure which is seen as the birefringent band in the polarizing microscope.

The progressive intermingling of filaments of the chromosomal spindle fibers effectively alters the organization of the mitotic spindle during mitosis.

4) In anaphase, the spindle fibers become shorter, without detectable change in thickness, until they are 5 to 10 μ long; at this time, the chromosomes stop moving and the spindle fibers fade and disappear. One cannot discern whether this disappearance is an actual disintegration or a hydration toward a refractive-index match that would make the fibers invisible in the Nomarski system.

The Nomarski system is clearly the instrument of choice for following refractive-index changes and movements of spindle fibers and other refractile bodies in the living spindle. So far, the pictures obtained support observations made with the electron microscope on carefully fixed material (*12*). They differ, however, in some interesting ways from the observations in polarized light. It has long been known from diverse material (*13*) that the birefringence of the spindle fiber is strongest near the kinetochore but fades and almost disappears toward the poles of the spindle. This was very clearly seen in *Haemanthus* (*13*). The polarized-light image suggested a "diffuse polar organization" difficult to reconcile with some aspects of chromosome movements. The fact that spindle fibers that are apparently uniform in their refractile properties extend from the kinetochores to the loosely organized spindle poles may at once simplify the interpretation of chromosome movements and place new significance on the findings with polarized light. The greater birefringence observed near the kinetochores (*13*) may be attributable, not to the refractile structures shown here, but to another component of the spindle that is unequally distributed or differentially organized along the spindle fiber. Alternatively, the drop in birefringence in the polar region could indicate that intermingling involves less orientation than a bundle of parallel filaments.

The intermingling of filaments from



Figs. 1–3. Mitosis in the endosperm of *Haemanthus katherinae*. Fig. 1. (*a*) Positive phase contrast. (*b*) The same cell photographed with differential interference contrast (Zeiss-Nomarski); details of spindle organization clearly visible. Fig. 2. Late anaphase. Fig. 3. Early telophase in the same cell. As anaphase progresses, fibers appear in the interzonal region which is transformed into the phragmoplast during telophase ($\times$ 1050; scale interval is 10 μ).

different spindle fibers serves as a partial explanation of certain phenomena which were difficult to reconcile with previous models of spindle structure, for example: the interference with anaphase movement of one chromosome when the movement of its neighbor is inhibited by a bridge, and the relative interdependence of individual chromosome movements in anaphase as compared with prometaphase.

ANDREW BAJER
Department of Biology, University of Oregon, Eugene
ROBERT D. ALLEN
Department of Biology, Princeton University, Princeton, New Jersey

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10. Three European companies, to our knowledge, already manufacture this system, although in the United States it is little used. The Zeiss equipment that we used was a prototype instrument made available to one of us (R.D.A.) through the kindness of Dr. H. Piller of Carl Zeiss (Oberkochen). It has high-quality polarizing and beam-splitting elements and is designed for use with strain-free planachromatic objectives and condenser.
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14. This investigation was conducted at Princeton University in May 1965; supported by grants NSF GB 3335 to A. Bajer and NIH GM 08691 to R. D. Allen. We thank Professor W. Jackson of the Department of Biology, Dartmouth College, Hanover, New Hampshire, for supplying us with plant material in the proper stage. Our studies were made possible by Professor Jackson's work on the developmental cycle of *Haemanthus* (NSF GB 705). Finally, we are grateful to Dr. H. Piller of Carl Zeiss (Oberkochen) for making available to us the prototype of a superb research instrument (Nomarski system).

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Gaseous-Diffusion Porometer for Continuous Measurement of Diffusive Resistance of Leaves

Abstract. *We describe a porometer that enables continuous monitoring of the stomatal diffusive resistance of leaves. The flux is measured of a gas—such as nitrous oxide—diffusing through a leaf that divides an enclosing chamber into two compartments. Nitrous oxide is added in known concentration to the airstream passing through the compartment on one side of the leaf and is recovered from the airstream passing through the opposite compartment. From measurements of the difference in concentration across the leaf and of the flux, the diffusive resistance of the leaf to N_2O is calculated; this value, adjusted for resistance external to the leaf, gives a continuous record of internal diffusive resistance. This record can be made simultaneously with measurements of transpiration and photosynthesis.*

In studying the fluxes of carbon dioxide and water vapor into and from leaves, one frequently requires a continuous, independent measure of stomatal aperture. Most conventional porometers have disadvantages (*1*). Diffusion porometers have the following advantages over the more commonly used viscous-flow porometers: operation can be continuous without disturbance of the gas and vapor concentrations around the leaf or of their fluxes; flow through the leaf is by molecular diffusion and thus similar in principle to that of water vapor and carbon dioxide; no external pressure is applied to the guard cells. The use of diffusion porometers using unusual gases, such as hydrogen (*1*), has been limited by difficulty in selecting a suitable gas that does not affect the plant and that can be continuously and inexpensively analyzed. Thus the flux of water vapor itself is often used to give a measure of diffusive resistance of the stomata (*2*, *3*).

Such estimates are complicated by doubts as to whether the resistance so measured arises entirely in the stomatal pore or is partly caused by resistances to vapor flow in the interfibrillar spaces of the mesophyll cell walls [it is generally assumed that resistance in the intercellular space is negligible (*4*)]. This uncertainty becomes of particular importance when independent estimates of stomatal aperture (such as by direct examination or by stomatal-imprint techniques) seem to indicate that, under apparently constant environmental conditions, the transpiration rate has varied without detectable compensating change in stomatal aperture. In such situations it is sometimes assumed that a significant source of resistance to vapor flow has developed in the cell walls but, because of difficulty in estimating stomatal diffusive resistances from measurements of stomatal apertures or by viscous-flow porometry (*5*), the assumption is difficult to substantiate. Thus a measurement of stomatal diffusive resistance is required that is unaffected by changes elsewhere in the path length of the water vapor.

We have developed a procedure in which the flux of N_2O is measured diffusing through a cotton leaf mounted in a specially designed chamber (*6*). The chamber permits independent measurements of transpiration from upper and lower surfaces of the leaf and, simultaneously, measurements of gaseous-diffusive resistances to water vapor and CO_2.

For measurements of stomatal diffusive resistance a controlled flow of N_2O, at known concentration, is introduced into the airstream flowing through the lower chamber. The N_2O diffuses through the leaf by way of the lower stomata, intercellular air spaces, and upper stomata and is then collected from the airstream passing through the upper chamber; the differential value (or absolute value in the upper airstream) is detected in an infrared gas analyzer sensitive to N_2O.

Table 1. Effects of four concentrations of N_2O (means of determinations at beginning and end of runs) on photosynthesis and transpiration. Light intensity: 0.2 cal cm^{-2} min^{-1} in the visible. VP, vapor pressure.

Concn. N_2O (vol %)	Duration (hr)	Net uptake CO_2 (%)	Background		Transpiration (%)
			CO_2 (vol %)	VP (mm-Hg)	
0	2.0	100	0.032	6.0	100
0.1	2.0	100	.032	6.0	107
.5	1.25	93	.032	6.0	97
1.0	0.55	102	.031	5.7	104
10.0	1.20	104	.029	5.4	112