A NEW METHOD OF POLARIZATION MICROSCOPIC ANALYSIS

I. Scanning with a Birefringence Detection System

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ABSTRACT

A new method of polarized light analysis is described in which a highly sensitive electronic detector specific for birefringence is used to identify the crystalline axes of an object and then measure its phase retardation due to birefringence. The microscopic system employed in the method consists of an electronic birefringence detection system (BDS), a microscope with strain-free lenses, and a driven stage for passing the specimen at appropriate velocities across the image of an aperture placed at the field stop and imaged in the specimen plane by the condenser. The detector registers retardations directly as voltage at a constant deflection sensitivity of *ca.* 1.1 v per angstrom unit over a range of 120 angstrom units. The basal rms noise level is 0.002 A for a spot 36 μ in diameter formed by a 95×, N. A. 1.25 objective pair, and increases in proportion to the reciprocal of the diameter of the scanning spot. The increase in noise with high resolution scanning can be offset by increasing the instrumental time constant, which is adjustable in decades between 0.004 and 0.4 seconds. A number of difficult problems in high extinction polarization microscopy are avoided by the use of modulated light and a rapid electronic detector. For example: (*a*) The measured distribution of birefringence is unaffected by the usual diffraction anomaly; therefore polarization rectifiers are not required. (*b*) The detector is selective for birefringence, so that there is no problem in separating contrast due to different optical properties (*e.g.* dichroism, light scattering). (*c*) The speed and sensitivity are both increased by between one and two orders of magnitude over that attainable by visual or photographic methods, thereby rendering a vast number of weakly birefringent, light-scattering, and motile objects readily analyzable for the first time with polarized light.

INTRODUCTION

The polarizing microscope is one of the few instruments with which cell biologists can hope to gain insight into the macromolecular organization of, and events in, living cells. In many cases it is more valuable to follow changes in macromolecular alignment revealed by birefringence in the living material, even at the restricted resolving power of light microscopes, than to rely wholly on high resolution electron microscopic images. Electron microscopic studies have the severe drawbacks that (1) they must be carried out on fixed material where the extent of damage cannot readily be determined by an independent method, and (2) fixation "freezes" the cell at some point

in time, thereby rendering it impossible to follow a sequence of physiological processes except by inferring the stages from still images.

Polarizing microscopes have been in use since the last century but, until about a decade ago, they were comparatively crude instruments. In fact, until recently, biologists have been obliged to make the best use of rather insensitive petrographic microscopes. In the early 1950's, Swann and Mitchison (21) and Inoué and Dan (10) independently suggested ways of improving the sensitivity of polarizing microscopes. The direction of these and later improvements has been to remove various causes of stray light in order to increase the extinction factor, and therefore the contrast, in the system. The "design for maximum sensitivity" of Inoué (9) has achieved a sensitivity gain of at least a factor of 10 over that previously attainable and seems to represent a practical limit to the sensitivity and speed that can be attained with available light sources and photographic detectors.

Until recently there has been a serious obstacle in the way of the simultaneous achievement of high sensitivity and high resolution with high numerical aperture lenses in polarized light. Rotation of the plane of polarization of incident linearly polarized light by curved lens surfaces not only produces the familiar polarization cross at the back focal plane of the objective, introducing significant amounts of stray light, but also causes a diffraction anomaly which introduces spurious resolution and contrast. The Airy diffraction image becomes altered to a clover-leaf pattern when viewed between crossed polars, and changes into a complicated elliptical pattern when ellipticity is introduced (12, 18).

A simultaneous solution to the diffraction anomaly and stray light problems caused by rotation at the lens' surfaces was found in "polarization rectifiers" developed independently by A. F. Huxley (6) and by Inoué and Hyde (11); these simple (but difficult to construct) elements return to parallel alignment the polarization azimuths of light waves passing through different parts of the specimen plane and back aperture of the objective. The rectifiers devised by Inoué and Hyde have been demonstrated to restore the normal Airy diffraction image so that the full resolving power of high numerical aperture lens systems can be utilized. The elegant study of Inoué and Sato (13) revealing the packing arrangement of DNA in chromosomes of insect sperm heads is clear testimony to the advantages of the improved contrast and resolution offered by a rectified lens system and to the remarkable skill and patience of these investigators.

High extinction polarization microscopy as exemplified by the work of the Inoué group may be the method of choice for moderately or highly birefringent objects, because, ideally at least, a single photograph may contain an enormous quantity of information. In practice, however, this information is incomplete without additional photographs taken at a series of specimen orientations and compensator settings. The collection of photographs must then be scanned densitometrically to determine some null point (extinction- or match-point). Thus the amount of time required to extract the desired quantitative data can be considerable.

One of us began several years ago to explore the pattern of birefringence in moving cells and streaming cytoplasm and immediately became confronted by three major obstacles. The first was a low signal/noise ratio in records of objects which were weakly birefringent and contained light-scattering inclusions. The second obstacle was lack of sufficient measurement speed; highest sensitivity measurements of retardations on film require photographic exposures of the order of 10 to 60 seconds. The time required to record fluctuations in birefringence during sporadic cytoplasmic streaming, for example, often exceeds the period of the changes themselves. The third obstacle was the poor resolving power imposed by the anomalous diffraction phenomena previously referred to.

The new method to be described seems to be the best solution now available for the technical problems mentioned above. In fact, it would seem to be the method of choice for highly polarized structures, such as contractile elements, mitotic spindles, and membranes, as well as objects with weak birefringence and contrast due to properties other than birefringence. Whether this method will replace the photographic method for objects as complex as the sperm heads studied recently by Inoué and Sato (13) depends chiefly on whether a scanning stage can be constructed with a sufficiently stable center of rotation.

THE BIREFRINGENCE DETECTION SYSTEM (BDS)[1]

The birefringence detection system (BDS) consists of a polarizer, electro-optic light modulator (EOLM), object, and analyzer, with an appropriate light source and photodetector (Fig. 1).

The polarizer and analyzer may be either high quality polaroid films or calcite prisms. We have followed Inoué's practice of using high extinction Glan-Thompson prisms, with stigmatizing lenses cemented to the analyzer surfaces to avoid astigmatism; but there would seem to be no reason why high quality polarizing filters could not be used as well.

The modulator is a birefringent plate, the principal axes of which are set at 45 degrees to the plane of incident polarization and can be rapidly interchanged (2–4). If the axes of the object are positioned *at the same angle*, the phase retardations introduced by the modulator (Δ_m in radians) and by the object (Δ_o) simply add, and the intensity of the light reaching the photodetector (I) is given by[2]

$$I = I_o \sin^2 \frac{\Delta}{2} = I_o \sin^2 \left(\frac{\Delta_m + \Delta_o}{2} \right) \tag{1}$$

I_o is the intensity of light passing parallel polars. When the fast and slow axes of the modulator are interchanged, the intensity becomes

$$I' = I_o \sin^2 \left(\frac{-\Delta_m + \Delta_o}{2} \right) \tag{2}$$

The change in intensity is thus

$$\Delta I = I - I' = I_o \left[\sin^2 \left(\frac{\Delta_m + \Delta_o}{2} \right) - \sin^2 \left(\frac{-\Delta_m + \Delta_o}{2} \right) \right] = I_o \, \Delta_m \sin \Delta_o \tag{3}$$

This change in intensity when the axes of the modulator are interchanged could be used as a measure of the phase retardation introduced by the object; however, it would also be affected by the incident light intensity (thereby changing I_o) by light-scattering or transmittancy changes on the part of the object, and by changes in the phase retardation Δ_m introduced by the EOLM. These potential difficulties are almost entirely avoided by adding a variable retardation plate or compensator, the axes of which are also aligned parallel to the axes of the modulator. The change in intensity on interchanging the modulator axes now becomes

$$\Delta I = I_o \sin \Delta_m \sin (\Delta_o + \Delta_c) \tag{4}$$

where Δ_c is the phase retardation introduced by the compensator. The system can now be used as a null device, with Δ_c adjusted to make ΔI equal zero; thus Δ_c equals $-\Delta_o$, independent of I_o and Δ_m.

The BDS as described so far already exhibits certain main features of the complete instrument:

(*a*) The use of a photoelectric detector increases the speed of measurement and brings the sensitivity very nearly to the theoretical limits imposed by statistical fluctuations in the light flux.

(*b*) The use of modulated light eliminates such instrumental difficulties as drift in DC amplifiers and variations in photomultiplier dark current, etc.

(*c*) The further addition of a null detection technique renders the system selectively sensitive to compensatable ellipticity from birefringence. Errors due to moderate amounts of stray light or to a wide range of fluctuations in lamp intensity, absorption, or light scattering in the specimen are avoided.

The technical problems of modulation and compensation have been solved in a simple and convenient way by the use of the Pockels electro-optic effect in a crystal of potassium dihydrogen phosphate. A thin basal section of this crystal is mounted with its Z axis parallel to the optical

[1] The components of the BDS are available commercially as follows: Photomultiplier voltage supply; Model 316, Baird Atomic, Cambridge, Massachusetts. Regulated power supply for modulator; Model 28, Lambda Electronics Corp., College Point, New York. Lock-in amplifier and waveform shaper; Princeton Applied Research, Princeton. Recorder; Power supply model 150–400, input coupling network and model 151 recorder, Sanborn Company, Waltham, Massachusetts. KDP electro-optic light modulators (EOLM); available from Baird Atomic and Isomet Corp., Palisades, New Jersey.

[2] The derivation of Equation 1 can be found in most text books of physical optics. For more complex calculations involving retardation plates at arbitrary angles, as in Equation 14, it is convenient to use the Poincaré sphere or such formalisms as the matrix calculus for optical systems described by Jones (15).

axis of the microscope. When an electric field is applied along the Z axis of the crystal, it becomes biaxial and acts as a retardation plate, the retardation of which is directly proportional to the applied field (2–4). By applying a combination of AC and DC voltages to the crystal, the functions of modulator and compensator are combined in this single element. The advantages of this system are considerable: there are no moving parts, the compensator scale is linear, and the electrical nature of the compensator lends itself readily to a rapid and completely automatic recording system (Fig. 1).

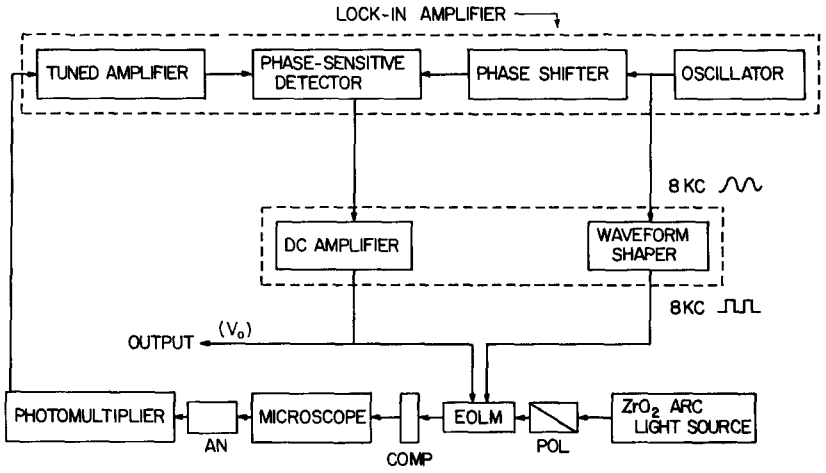

FIGURE 1. A block diagram of the optical and automatic recording systems. *An*, analyzer; *comp*, compensator; *pol*, polarizer.

THE AUTOMATIC RECORDING SYSTEM

In the system as described so far, the introduction of a birefringent (phase-retarding) object leads to a fluctuation in the transmitted light intensity at the frequency of modulation. As stated previously, the magnitude of this AC signal is determined by the retardation or path difference introduced by the object; the direction of the slow axis determines whether the signal is in phase with the modulating voltage or 180 degrees out of phase with it. This AC signal is then fed into a phase-sensitive detector or lock-in amplifier, which produces a DC voltage proportional to the amplitude of the AC signal. The sign of this DC voltage depends on whether the signal is in phase or 180 degrees out of phase with the modulating voltage. This voltage is then amplified to produce the final output (Fig. 1).

The BDS can be characterized at any given time in terms of two numbers. The first, β is the electro-optic coefficient, or ratio of the phase retardation induced in the EOLM to the voltage applied:

$$\beta = \frac{\Delta_c}{V_c} \tag{5}$$

The value of β is a constant for the type of EOLM crystal used, but may vary slightly with the particular electrode configuration employed.

The second number, K, is a measure of the DC voltage V_o at the final output of the lock-in amplifier produced by a net phase retardation Δ in the system:

$$K = \frac{V_o}{\Delta} \tag{6}$$

The value of K is proportional to light intensity, the sine of the modulation amplitude Δ_m (Equation 4), the photomultiplier sensitivity, and the gain of the amplifier.

When a birefringent object with a phase retardation Δ_o is introduced into the system, a voltage $K\Delta_o$ appears at the output of the DC amplifier. This voltage is applied to the EOLM as negative feedback; that is, the phase retardation it induces in the EOLM (which is now acting as a compensator) is opposite in sign and nearly equal to

that introduced by the object. Closing the feedback loop therefore makes the BDS an automatic null-seeking system, the output of which is a new voltage, V_o, related to the system as follows: the phase retardation induced in the EOLM is

$$\Delta_c = \beta V_o \tag{7}$$

The net phase retardation is therefore

$$\Delta = V_o - \Delta_c = \Delta_o - \beta V_o \tag{8}$$

The number K was earlier defined as a measure, at a given time, of the DC output of the lock-in amplifier produced by the net phase retardation in the system. This is the same voltage referred to as V_o:

$$V_o = K\Delta = K\Delta_o - K\beta V_o \tag{9}$$

Hence

$$V_o = \frac{K\Delta_o}{1 + K\beta} = \frac{\Delta_o}{\beta}\left(\frac{K\beta}{1 + K\beta}\right) \tag{10}$$

Thus, if $K\beta$, referred to as the loop gain, is very large compared to one, the factor in brackets is essentially unity, and the output voltage is directly proportional to the phase retardation Δ_o; the coefficient of proportionality is simply $1/\beta$, a constant since it is a characteristic of the EOLM alone. The actual numerical value of K, which depends on so many instrumental factors, is unimportant.

In high resolution scanning with small spots, or other situations in which the amount of available light reaching the photomultiplier may be small, it may happen that K, and therefore the loop gain $K\beta$, falls to such a value that $K\beta$ $(1 + K\beta)$ differs significantly from unity. In this case, it is necessary only to measure the loop gain and apply a correction. This can be done most conveniently by disconnecting the EOLM from the lock-in and applying to it a known voltage V_s instead. Then

$$\Delta_c = \beta V_s \tag{11}$$

and

$$V_o = K\Delta_c = K\beta V_s \tag{12}$$

or

$$K\beta = \frac{V_o}{V_s} \text{ (open feed-back loop).} \tag{13}$$

In practice, $K\beta$ varies from about 10 (where the correction is 10 per cent) to over 100.

Since the output is already in the form of a voltage, it is a simple matter to divert some fraction of it to an oscilloscope or other recorder.

THE MICROSCOPE

The present instrument with which this method is used is an inverted microscope similar to the arrangement described by Inoué (9). Filtered monochromatic or heterochromatic light from a mercury arc or a zirconium oxide–concentrated arc source is focused on a pinhole and then projected by means of a 25 mm cemented achromatic lens to form an image of the source on the entrance pupil of the condenser of the microscope. The latter is actually a microscope objective, one of a pair chosen for freedom from strain birefringence. Scanning apertures are placed at the field stop and imaged in the specimen plane by means of the condenser.

After passing the scanning aperture, light traverses a polarizer, EOLM, and compensator goniometer which can accomodate either a Köhler $\lambda/10$ to $\lambda/30$ mica plate (17) or a quarter-wave plate. Light leaving the microscope proper passes through an analyzer before being divided: 10 per cent to the eye and 90 per cent to the photomultiplier. The specimen can be visualized by the addition of unmodulated far-red light from a filtered second light source; this light can be applied either from the level of the stage as a dim direct illumination, or it can be directed through the lenses of the microscope by introducing a tilted coverglass as a reflector in the light path.

The method can be applied to simpler microscopes as long as the lenses are free from strain and the EOLM is placed in a firm mount which can be leveled. High quality film polarizers can be substituted for Glan-Thompson prisms without appreciable loss of sensitivity.

Alignment

Optical alignment consists of the usual steps required to obtain Köhler illumination and a centered, symmetrical polarization cross at the

back aperture of the objective. Once the lenses are centered in their quick change carriers, they are removed and the EOLM inserted into the light path. The latter is leveled to obtain the best extinction position by eye. Precise leveling is carried out later using the detector *before* the lenses are replaced.

Electronic adjustments consist of the following:

(*a*) With the feedback loop open, the lock-in amplifier is tuned to respond with a maximal output signal (V_o) to a small retardation introduced by a mica plate rotated about a degree from extinction position.

(*b*) The mica plate is then removed, the light beam interrupted, and the DC bias control on the lock-in amplifier adjusted to zero.

(*c*) The lock-in amplifier is then used (with the feedback loop broken between the DC amplifier and EOLM) to perform the fine adjustment leveling of the EOLM.

(*d*) The feedback loop is closed and a small retardation again added in order to measure the loop gain ($K\beta$). This can be done either by measuring with the feedback loop open, then closed; ($K\beta = V_o$ open$/V_o$ closed), or by comparing V_o (open loop) with a reference signal (V_s).

The object must be rotated until its maximum retardation is registered. At this setting, its crystal axes will be parallel to the axes of the modulator.

Calibration

The range for which the present detector was designed is so small that an accurate calibration requires an extremely sensitive goniometer. That used in the present instrument is accurate to 0.005° for small angles and will accommodate either a thin mica compensator or a quarter-wave plate.

The most convenient way to calibrate the detector is with a quarter-wave plate rotated a few degrees about its extinction point. Since the crystal axes are far from parallel with the modulator axes in this case, Equations 1 to 4 no longer apply. Nonetheless, it can be shown[2] that there still exists a compensator setting for which there is no change in transmitted intensity when the modulator axes are interchanged. If the quarter-wave plate is rotated by an angle θ from its extinction point, the phase retardation ϕ of the automatic compensator is given by

$$\tan \phi = \frac{\sin 2\theta}{\cos^2 2\theta} \tag{14}$$

For small angles (less than 4° of arc) the following approximation is accurate to 1 per cent:

$$\phi = 2\theta = 2\theta\pi\Gamma/\lambda \tag{15}$$

PERFORMANCE

Sensitivity

The deflection sensitivity of the birefringence detection system, with or without lenses in place, is approximately 1.1 v per angstrom unit of retardation.[3] Response is linear over the entire range of ±60 angstroms. (One angstrom unit of retardation is equal to a phase retardation of 1.15×10^{-3} radians at the wavelength of the mercury green line, 5461 A.) A variation in deflection sensitivity of about 10 per cent was found moving the EOLM around so that the light beam fell on different parts of the electrode configuration. Once the position of the EOLM was fixed, however, the response was linear and the deflection sensitivity constant at high loop gains (Fig. 2). (It will be recalled that for small scanning areas or low light intensity the loop gain must be measured and a correction applied.)

Noise, Filtering, and Time Constants

The smallest retardation detectible, or the ultimate sensitivity, depends on the noise level; this in turn depends on the light intensity, stray light, scanning aperture size, and electronic filtering employed. The minimum noise level attained by careful alignment of all components was 2 mv rms or about 0.002 angstrom unit when the entire 9 mm field lens aperture was imaged to form a spot 36μ in diameter in the specimen plane, and when the time constant due to filtering was 0.4 second. The smallest spots employed by us with the present instrument have been 1.3 microns in diameter in the oil immersion field.

[3] The KDP modulators respond at a ratio of 3.5 per angstrom unit. The deflection sensitivity differs from this value because a voltage divider is used at the output.

For the same degree of filtering, the rms noise level was less than 0.1 angstroms (Fig. 3).

A choice of resistance-capacitance filters provide time-constant adjustment in decades from 0.004 to 0.4 second. Tests were performed in which sheets of strained glass were suddenly introduced into the light path to confirm the expectation that the response to a step function was determined only by the RC filtering employed.

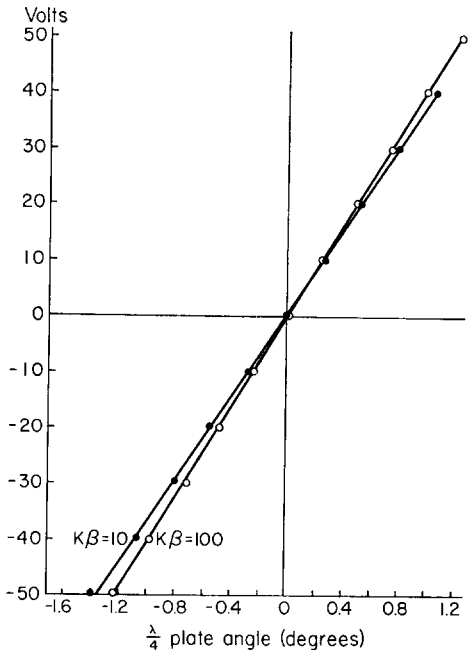

FIGURE 2 An experimental plot of the deflection sensitivity of the birefringence detection system at loop gains of 100 and 10, showing the necessity of applying a correction at low loop gains.

Resolving Power

It is important to know the resolving power of any microscope used for biophysical investigations. This is particularly essential for any polarization microscopic method, because Inoué and Kubota (12, 18) have shown that a diffraction anomaly referred to earlier introduces spurious resolution and contrast into images in unrectified polarizing microscopes.

In the present method, birefringence is detected not in the distribution of image contrast but coded by AC modulations in light transmitted through a restricted specimen area. Therefore the question of resolving power must be considered differently. First, it must be known whether the demagnified image of the scanning aperture correctly samples the desired specimen area, that is, whether the demagnified spot is the classical Airy disc or some other pattern of energy distribution. Second, it must be known whether the modulated light traversing the object through the center of the lens carries the same information with respect to birefringence as light taking the longer path through the edges of the objective, especially in the bright quadrants where depolarization occurs.

As to the question of the structure of the image of the scanning aperture, one can see that the diffraction image in a polarizing microscope lacking an analyzer is virtually the normal Airy pattern. Thus approximately the normal 84 per cent of the energy from the scanning aperture passes through the demagnified image of the spot in the specimen plane. The remaining 16 per cent is present in the peripheral diffraction rings. It is only when the analyzer is added that the clover-leaf pattern described by Inoué and Kubota appears in conjugate planes *behind the analyzer*.

The question of homogeneity of information traversing different parts of the lens at different angles has been answered experimentally in the following way. Two coverglasses, one of which had been strained, were placed between matched strain-free high-dry (N.A. 0.6) objective lenses to simulate usual observation conditions, and were caused to revolve by driving the stage with an electric motor. An image of the back focal plane of the objective was then scanned by moving the photomultiplier, covered by a small fixed aperture, over the image. The signal amplitude and phase were equal to within a few per cent in parts of the aperture. However, the noise level did increase in the four quadrants representing the bright areas in the polarization cross visible when the input voltage to the modulating crystal was disconnected.

While the amplitude of an AC signal does not vary across the back aperture of the objective, there are three conditions in which the DC level at any point in the back aperture may differ from the average: (*a*) If the EOLM is slightly tilted, or its coverglass strained, this will show as one or more gradients in DC level across the aperture. (*b*) If the lenses contain any strain birefringence, this will be detected in scanning the back aperture

over the area of strain. (*c*) If a mica compensator is present in the system, the DC levels in opposite quadrants will vary in the same direction, and those in adjacent quadrants will vary in opposite direction. This is a consequence of the sensitivity of the detector to rotation of the analyzer if a birefringent object is present with its axes at some angle other than 0° or 45° to the plane of polarization.

Response Specificity

The BDS was designed to respond only to compensatable ellipticity introduced by birefringent objects. Tests were carried out to determine to what extent the system might respond to extraneous factors in the environment.

STRAY LIGHT: Room light allowed to leak into the photomultiplier housing, or unmodulated light introduced into the optical system by reflection in moderate amounts had almost no effect on the deflection sensitivity. However, they did increase the noise level. In practice, care must be taken not to saturate the photomultiplier. Short of saturation, stray light is not likely to introduce serious errors.

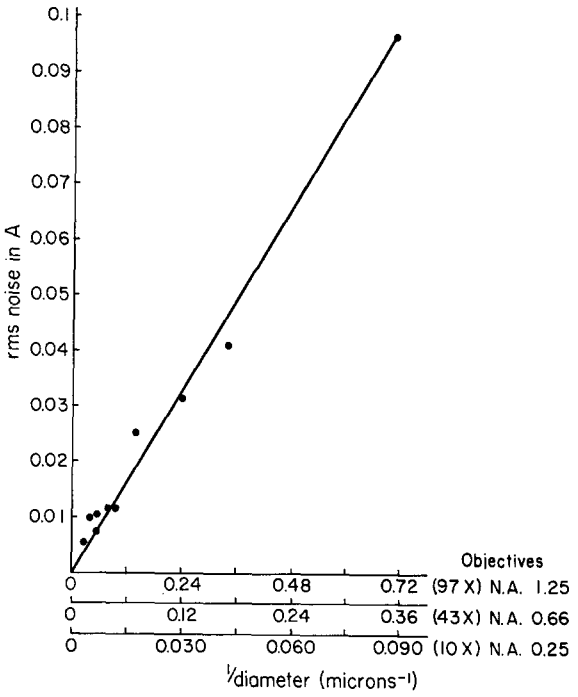

FIGURE 3 An experimental plot of the rms noise level in angstroms (ordinate) as a function of the reciprocal of the diameter of the scanning spot imaged in the oil immersion, high dry, and low power fields (paired objectives).

In practical terms this information indicates that it is safer with this method to move the object in scanning rather than the light beam. It is also safer to avoid the use of additional compensators in the system, even though changes in DC level in adjacent quadrants average out across the whole objective aperture.

It seems safe to conclude that the resolution of scanning polarization microscopy by this method is diffraction-limited without the anomaly problem that has plagued high extinction methods. Thus rectifiers, which are difficult both to construct and to obtain commercially, are not required, and the entire working aperture of high-dry and oil immersion lenses may be utilized for their full resolving power.

OPTICAL ROTATION: Rotation of the plane of polarization before the EOLM causes only a very minor reduction in deflection sensitivity over an arc of 3–4° and increases the noise level somewhat. Rotation of the analyzer may or may not influence the signal level, depending on the optical elements present in the light path. If no birefringent object is present in the light path, the detector does not respond to analyzer rotation.

On the other hand, if a retardation plate follows the EOLM, the system as a whole responds to rotation of the analyzer. For example, with a $\lambda/30$ plate the system responded with a deflection of 1 v per 0.08°. The significance of this fact as far as the method is concerned is clear: compensators other than the automatic one (the EOLM) should be avoided in order to keep the system as a whole selectively sensitive to birefringence alone (Fig. 4).

When a quarter-wave plate (with its crystal axes parallel to the plane of polarization) follows the EOLM, these two elements in concert act as a polarization azimuth vibrator in a manner analogous to a Faraday rotatory cell. Under these conditions the system responds both to rotation and to birefringence, the rotation deflection sensitivity being 1 v per 0.028°. The practical significance of this fact is that the quarter-wave plate can be used for calibration only, and not as a bias retardation in scanning birefringent objects.

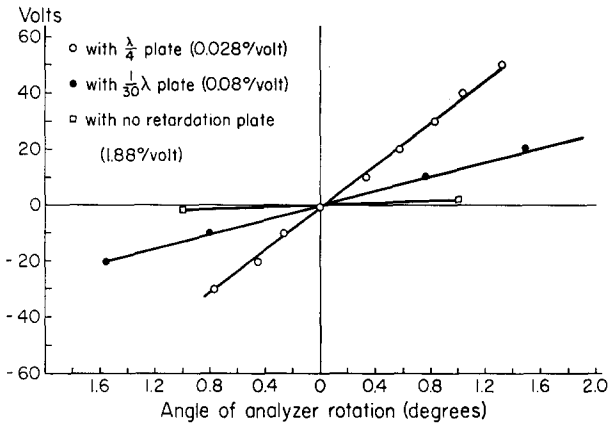

FIGURE 4 An experimental plot of the response of the entire system to rotation of the analyzer. (□) EOLM alone in the system, (●) mica compensator ($\lambda/30$) present, (○) quarter-wave plate present.

REDUCED TRANSMITTANCE DUE TO ABSORPTION OR LIGHT SCATTERING: The null-seeking feature in the BDS design eliminates the possibility that an absorbing or light-scattering body could be "mistaken" for a birefringent one by the system as a whole. That is, neither of these presents compensatable ellipticity.

On the other hand, a given signal level will diminish if the intensity of the modulated light reaching the photomultiplier is significantly reduced, either by absorption or light scattering on the part of the specimen or by gross changes in the intensity of the source. Thus while the system is incapable of false detection of birefringence, the investigator must check the loop gain (see previous section on the automatic recording system) and correct deceptively small signals.

DICHROISM: Dichroic objects which are not birefringent should act in the compensator-free system as rotators and therefore not be detected unless a mica compensator or quarter-wave plate follows the EOLM. In practice, most biological objects which are dichroic are birefringent as well. Diochroism alone is detectable if a $\lambda/4$ plate is inserted after the EOLM and the analyzer of the microscope is removed, allowing the dichroic object to function as the analyzer.

CHANGES IN AMBIENT TEMPERATURE: Changes in room temperature, air currents, etc. cause a measurable amount of drift (about 1 v/hour) in the signal level. This is caused by thermally induced movements on the part of the light source and/or EOLM. Electro-optic light modulators made from potassium dihydrogen phosphate must be mounted so that the Z axis of the crystal and the optical axis of the microscope are precisely paraxial. If either the light source or the EOLM moves for any reason, the effect is the same as tilting a Berek or Ehringhaus compensator in the light path.

Care must be taken to avoid the introduction of strain birefringence into the lenses. It is important to point out that merely touching one of the lenses with a finger is sufficient to introduce a transient signal.

DISCUSSION

An investigator contemplating the analysis of birefringence in a biological object has a choice of

three possible photometric methods: visual, photographic, and electronic. Each has certain strengths and weaknesses which have to be taken into account along with the nature of the specimen and the requirements of the problem.

Visual Detection and Measurement of Birefringence

The dark-adapted eye is for many purposes an efficient detector of the contrast produced by a highly transparent birefringent object in a high extinction polarizing microscope (8, 9). Even without a compensator, the eye can detect retardations in the angstrom unit range, and according to Inoué (9) 0.1 angstrom unit can be detected by means of a Köhler compensator (17) and a crystal comparator imaged in the field beside the specimen area. Under best conditions the time required for a single measurement of retardation at some point in the image is of the order of seconds or tens of seconds. Usually, minutes are required for a series of measurements which can be relied upon.

While visual inspection of a microscopic object is an essential preliminary for measurement of its pattern of birefringence, visual measurements themselves are subject to many difficulties. The following are some situations in which the investigator must take recourse to either photographic or electronic detection methods.

(*a*) The sensitivity may be insufficient for the accuracy desired.

(*b*) The object may show motility or fluctuations in birefringence which are too rapid to be measured visually.

(*c*) The pattern of birefringence or structural detail in the object may be too complex to be measured in the desired number of points in the image.

(*d*) The object may display contrast due to optical properties other than birefringence; light scattering in particular interferes seriously with the setting of a compensator for match or extinction points.

Photographic Detection and Measurement of Birefringence

It is well known that photographic images can be used to perform the same functions as the eye in finding the extinction or match points of parts of the field. It i also possible to use the contrast in photographic density in two images taken at balancing positive and negative compensator settings as a direct measure of phase retardation. The latter method is, however, susceptible to experimental errors due to the well known pitfalls of photographic photometry (19).

The most careful study to date using film photometry has been that of Inoué and Sato (13), who avoided using density contrast as a direct measure of phase retardation, and who instead studied the change of contrast in photographs taken at a series of specimen and compensator orientations. In this way they were able to work out in detail the azimuth orientation and the phase retardation at all points within the specimen image.

The exposure time required for a single 35 mm photograph with the compensator set for maximum contrast in a high extinction system is of the order of 10 to 60 seconds. There is thus a serious limitation on photographic as well as visual studies of objects which may move or exhibit fluctuating birefringence.

While photographic registration of the contrast due to birefringence provides permanent records and has a number of other important advantages, there are several experimental situations in which photographic methods, as well as visual methods, are inappropriate.

(*a*) The sensitivity, approximately the same as that for visual methods, may be insufficient or limited by photographic grain "noise."

(*b*) The object may move too rapidly to be photographed or its birefringence may fluctuate. Such a fluctuation may not even be detected unless it happens to be periodic and examined with stroboscopic illumination.

(*c*) If the object absorbs or scatters light, contrast due to birefringence may be obscured in part or entirely in photographs.

Electronic Detection and Measurement of Birefringence

Visual and photographic methods depend on the simultaneous presentation to the detector of a brightness or intensity difference in the image. Electronic detection and measurement methods, in contrast, depend on the "memory" of the detector for intensities at the same point in the specimen or scanning area when subjected to a known sequence of phase retardations.

It is possible to use either a simple photocell or

a more sensitive photomultiplier photometer to measure the extinction point of an object and its background when a compensator is rotated. Used in this way, a photoelectric detector is merely an objective substitute for the eye, performing the same functions at a small gain in sensitivity and at a probable loss in speed.

A photoelectric detector can also be used directly to detect intensity changes due to object birefringence changes. This method was perhaps first used by Schmitt and Schmitt (20) to determine whether a birefringence change accompanied the passage of the nerve impulse. This method is, of course, sensitive to intensity changes due to other causes.

More recently, Eberstein and Stacey (5) have used a fixed $\lambda/4$ plate and a rotating analyzer to measure changes in phase retardation with smooth muscle contraction. The smallest retardation detectable was about 20 angstrom units.

Allen and Rebhun have suggested a simple modulation method employing a rotating tilted compensator to detect and measure weak birefringence in light-scattering objects (1). The sensitivity is comparable to that of visual and photographic measurements for objects 10 microns in diameter and larger, but the time required for a measurement is of the order of tenths of seconds to seconds, depending on the noise level. The advantage of their method was in the avoidance of much light-scattering noise.

The photoelectric methods mentioned so far appear to be the only ones that have been applied to microscopic and macroscopic analysis of biological objects. In general, the speed and sensitivity of these methods do not represent any significant improvement over visual and photographic methods.

Other arrangements have been described which have either not been suitable for microscopy, or which have simply not been so applied. West and Jones (24) have reviewed the literature on such devices. One early application of a modulation technique was the photoelectric method of Kent and Lawson (16) in which elliptically polarized light was converted to circularly polarized light by a variable compensator so that the amplified photocell signals set up by a revolving analyzer were not detectable aurally by headphones. The effects of stray light on the system were said to be negligible.

Even in the early work of a quarter of a century ago, it was clear that the sensitivity of photoelectric methods would surpass that of other methods. Ingersoll (7) described a photoelectric method capable of detecting 0.05 angstrom unit of retardation, a value comparable to the sensitivity of the best visual compensators (*cf.* Jerrard, reference 14).

The use of modulation techniques to analyze ellipticity has been greatly aided by the introduction of crystals displaying the Pockels electro-optic effect (2–4). Within the last few years, Takasaki has introduced a series of interesting and useful instrument designs based on ADP (ammonium dihydrogen phosphate) crystals (22, 23). Among these, his birefringence analyzer is the most interesting in the present connection, for it is potentially of use as a birefringence detector for a polarizing microscope. In this system, the object stage and a Soleil-Babinet compensator are both driven by servo motors so that the object is rotated to its azimuth orientation where its retardation is measured by the Soleil-Babinet compensator. The Takasaki system is similar to that reported here in that a detector signal is fed to servo motors which in effect serve as phase-sensitive detectors. While the range of such an arrangement is considerably broader than that of the birefringence detection system described here, the speed and sensitivity are bound to be both considerably less; the presence of moving parts also introduces increased possibilities for error.

To our knowledge, the birefringence detection system described here is the first completely automatic device of its kind applied to polarization microscopy. As a microscopic system for biophysical research it possesses a number of important advantages:

(*a*) A selective sensitivity to birefringence of about 1.1 volt/angstrom unit.

(*b*) Filtering time constants (τ) from 0.004 to 0.4 second (or longer if needed).

(*c*) A low basal noise level (0.002 rms angstrom units for large spots, τ = 0.4 second[4]).

(*d*) The full resolving power of high numerical aperture lenses can be utilized without recourse to polarization rectifiers.

(*e*) The use of a scanning system avoids exposing light-sensitive specimens to high levels of illumination.

[4] As with any instrument of this type, the noise level varies with the reciprocal of the square root both of the photon flux and of the time constant.

Present Limitations and Possible Future Applications

HIGH RESOLUTION SCANNING: The present method can, in principle, be used to scan the smallest resolvable specimen area (1 Airy unit in diameter) and determine the average axis orientation and weighted mean retardation in that area. Whether a complex object could be scanned as successfully by this method as Inoué and Sato were able to do with high extinction photographic methods depends on the extent to which two shortcomings in the present instrument can be overcome in the future.

It was pointed out earlier that the crystalline properties of the present EOLM cause a gradual drift over the periods of time that would be involved in scanning of the order of a hundred points in a complex object. Modulators made from cubic crystals are expected to be available in the near future; with these substituted for the present KDP crystal, the noise level should decrease considerably and drift should no longer be a problem.

With a superior modulator the limit to both speed and sensitivity will be photon noise. Further gains in these properties will be possible when brighter light sources (*e.g.* lasers) become generally available. For example, the noise level will decrease as the square root of the intensity.

If a complex object is to be scanned for its axis orientation and retardation, a stage must be constructed with an absolutely stable center of rotation (constant to 0.05 micron) and capable of being moved along perpendicular X and Y axes by increments of 1 Airy unit (0.2 micron for the oil immersion system). While these specifications may be too much to hope for, it should be possible to select a stage which would permit scanning with apertures of submicron dimensions. To the present, our own work has involved mostly low resolution scanning of objects with known axial orientation.

TIME-DEPENDENT BIREFRINGENCE CHANGES: The high speed with which the detector can respond (95 per cent full response in 0.01 second) if noise in the angstrom unit range can be tolerated should make it possible to follow fairly rapid changes even in muscle contraction. The system has already been used to follow fluctuations in birefringence associated with sporadic or cyclic cytoplasmic streaming. When the drift problem has been eliminated by a better modulator, it should be possible to record the very gradual development of birefringence in parts of embryos or organ cultures.

Other possible applications of the method involving time-dependent changes in birefringence are: measurement of flow birefringence in dilute solutions or at very low velocity gradients, quality control of lenses for polarizing microscopes and other optical instruments, the study of photoelasticity in plastics, synthetic substances, and even components of living cells.

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