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Modulated Light Methods in Polarized Light and Interference Microscopy

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The light microscope is used today principally as a magnifier for observing and photographing cells, tissues, microorganisms, and various other small objects. As everyone knows, a good microscopist strives to achieve in his photomicrographs what has come to be called "good image quality". In other words, he attempts to capture and record as much information about the specimen as possible. As is well known, the factors which limit the amount of detailed information in an image are (1) the numerical aperture of the optical system, which determines the lateral resolving power, and (2) the efficiency of the contrast-generating system and its specificity for a particular optical property.

Contrast in all microscopic images depends upon well-understood light-matter interactions, such as absorption, refraction, diffraction, double-refraction (birefringence), optical rotation, fluorescence, polarized absorption (dichroism) and polarized fluorescence. It is now well established that each of these light-matter interactions, which I shall later refer to only as "optical properties", may impart extremely useful information to cell biologists, especially those who are interested in the function of macromolecules in cells and tissues.

Within the last quarter of a century a number of important specialized microscopes have been produced which permit microscopists to emphasize contrast in the image due to one specific optical property. The phase-contrast, interference, and fluorescence microscopes and the microspectrophotometer are familiar examples. Polarizing and dark-field microscopes are older, but perhaps less familiar instrments of this type.

In the case of each of these specialized microscopes, contrast due to a single optical property is enhanced, but the contrast never becomes highly specific for a particular optical property. As a consequence, precise quantitative microscopic measurements are almost impossible. For example, in microspectrophotometric measurements of light absorption, errors may also be due to refraction, light scattering, and dichroism. Similarly in birefringence measurements light scattering, absorption, and refraction may introduce errors. The multiplicity of contrast sources thus plagues virtually every kind of physical microscopic measurement.

Considering the fact that most of the information we have about the form and function of cells has come to us through the microscope, it behooves us not to throw away needlessly any potentially useful and interpretable information just because of the purely technical problem posed by the multiplicity on contrast sources. A list of important information that can be obtained from physical microscopic measurements is already impressive: it includes the dry mass of single cells or their inclusions, organic matter concentration, molar concentrations of specific substances, the orientation of fibrils, membranes or molecules below the resolution limit of the microscope lenses, changes in macromolecular conformation, the orientation of chemical bonds, the localization and concentration of fluorescent stains, antibodies, etc. The rewards would therefore be significant if a way could be found for making precise measurements of the optical properties of microscopic specimens without recourse to the usual separation of image contrast into its component causes.

I am very pleased to report that considerable progress has been made toward the development of a universal measuring microscope which in effect allows the microscopist to encode a selected optical

property for registration as an electrical signal, which can be used for point measurements, one- or two-dimensional scans of the image, measurements at one or more points in the image through time, and spectral scans. An instrument has been produced for us by the Princeton Applied Research Corporation with the cooperation of the Carl Zeiss works in Western Germany through a Research Contract with National Aeronautics and Space Administration. The Par-Zeiss Microscope System, as it will be called, is an integrated optical-electronic system embodying an entirely new approach to quantitative physical microscopic measurements which entirely avoids the use of image contrast in the conventional sense as a measure of any optical property. Instead, each optical property may be selectively encoded as an intensity modulation pattern through the use, for each optical property, of an unique arrangement of auxilliary optical devices in the microscope. Each optical property is measured electrically on a linear scale with a maximum allowable error of one percent of whatever full-scale deflection sensitivity is in use. The advantages of this approach are that (1) measurements may be made very rapidly (with response times ranging from one msec to ten seconds), (2) the noise level is extremely low, so that very small effects can be detected, and (3) there is no loss whatever in lateral resolution made possible by the microscope lenses. This last statement is true even in polarized light microscopy where ordinary images are distorted by anomalous diffraction.

The principles basic to all of the methods that can be used with this instrument are best described for the case of the Birefringence Detection System, published last year by Allen, Brault and Moore in "The Journal of Cell Biology". In this system, light from a suitable source is passed through a polarizer, an electro-optic light modulator (EOLM), a specimen in an optical system free from strain, and an analyzer,

and is detected by a sensitive photomultiplier.

The EOLM is the most essential element in the system, since it serves not only as the light modulator for encoding the birefringence information from the specimen as a photocurrent amplitude modulation, but also as an automatic compensator for measuring phase shifts or phase retardations by applying electrically-induced phase shifts of opposite sign. The EOLM is a Z-cut crystal of potassium dideuterium phosphate which acquires new crystalline axes; that is, it becomes birefringent, when a voltage is applied to its optical surfaces. The induced birefringence is known as the Pockels effect, and its value in this application is its strict linearity and known wavelength dependen

The operation of the system is best shown with a diagram (Fig. 2). As oscillator drives the EOLM with square-waves of AC, forcing it to introduce square-waves of phase shifts on both sides of zero. When a phase retarding specimen is added to the optical system, the total retardation is no longer symmetrical about zero, so that the rectified square-waves of photocurrent from the photomultiplier develop a repetitive asymmetry, which in effect is a photocurrent amplitude modulation at the fundamental frequency of the oscillator. The lock-in amplifier, which is a phase- and frequency-sensitive device deriving its reference signal from the oscillator driving the EOLM, responds to this photocurrent modulation with a large DC voltage, which is returned to the EOLM as negative feedback to return the total retardation to zero. This servo is linear by virtue of the linearity of the Pockels effect, and the instrument is designed to operate with uniform error of one percent.

The microscope is a Zeiss Photomicroscope I modified to accomodate the modulator assembly, apertures, slides, filters, mirrors, etc. which are needed for this method. The design shown in Figure 3 allows us to

illuminate the specimen with full-field red unmodulated light while scanning it with a microbeam of short-wavelength light with which measurements are made. Light from a zirconium oxide arc is first filtered for the desired spectral region, then passed through the modulator assembly. The microbeam is formed by a slit or circular aperture imaged first in the plane of the rear diaphragm then again in the specimen plane. A dichroic mirror mixes short-wavelength microbeam energy with the long-wavelength light used to permit an observer to locate the exact position of the microbeam relative to the specimen and detect any form changes or movements of the specimen during measurements. The observer sees, or may record photographically, a blue or green spot (the focused microbeam) in a red field. A dichroic beam splitter in the tube-head sends the microbeam energy to the photomultiplier and the red illumination to form an image of the specimen.

With this system, it is possible to make spot measurements in the specimen and record changes with time; or, the specimen may be slowly drawn across the microbeam to provide a one or two dimensional scan. Mr. Richard Burger in my laboratory has designed a simple electromechanical device which provides linear motions which are reproducible to very nearly the resolution limit of the microscope. The smallest phase retardation that can be measured is approximately one millionth of a wavelength of light, or a distance which is small compared to interatomic distances. The limitation on the absolute sensitivity has proven to be photon noise; that is, statistical fluctuations in the stream of photons striking the photomultiplier. The significance of this fact is that substantial improvements in performance of instruments of this type can be attained only by increasing the brightness of the light source.

The instrument which has made such sensitive measurements possible has been in our laboratory for only a short time. However, it is on loan to the company which developed it while I attend two meetings. It is now on exhibit at this meeting. I shall not give you any detailed information on the performance of the instrument since you may see it in operation and examine the preliminary specifications yourselves.

Instead I should like to [illegible] some of the possible types of measurements that can be done with [illegible] system. These fall into three classes. In the first class (Figure 4) are the functions associated with polarized and interference microscopy. Note that if a quarter-wave plate follows the EOLM, the instrument records not birefringence but optical rotation. It is still sensitive to birefringent objects, but only at certain angles at which these objects introduce a spurious rotation. We have found that it is possible to measure rotation and birefringence independently of one another if a second EOLM is added and two lock-in amplifiers at different frequencies are used. One channel allows the experimentor to remove any spurious rotation due to the birefringent object and the other allows a measurement of the residual rotation which is real. This is apparently the first system with which it has been possible to measure optical rotation in microscopic objects.

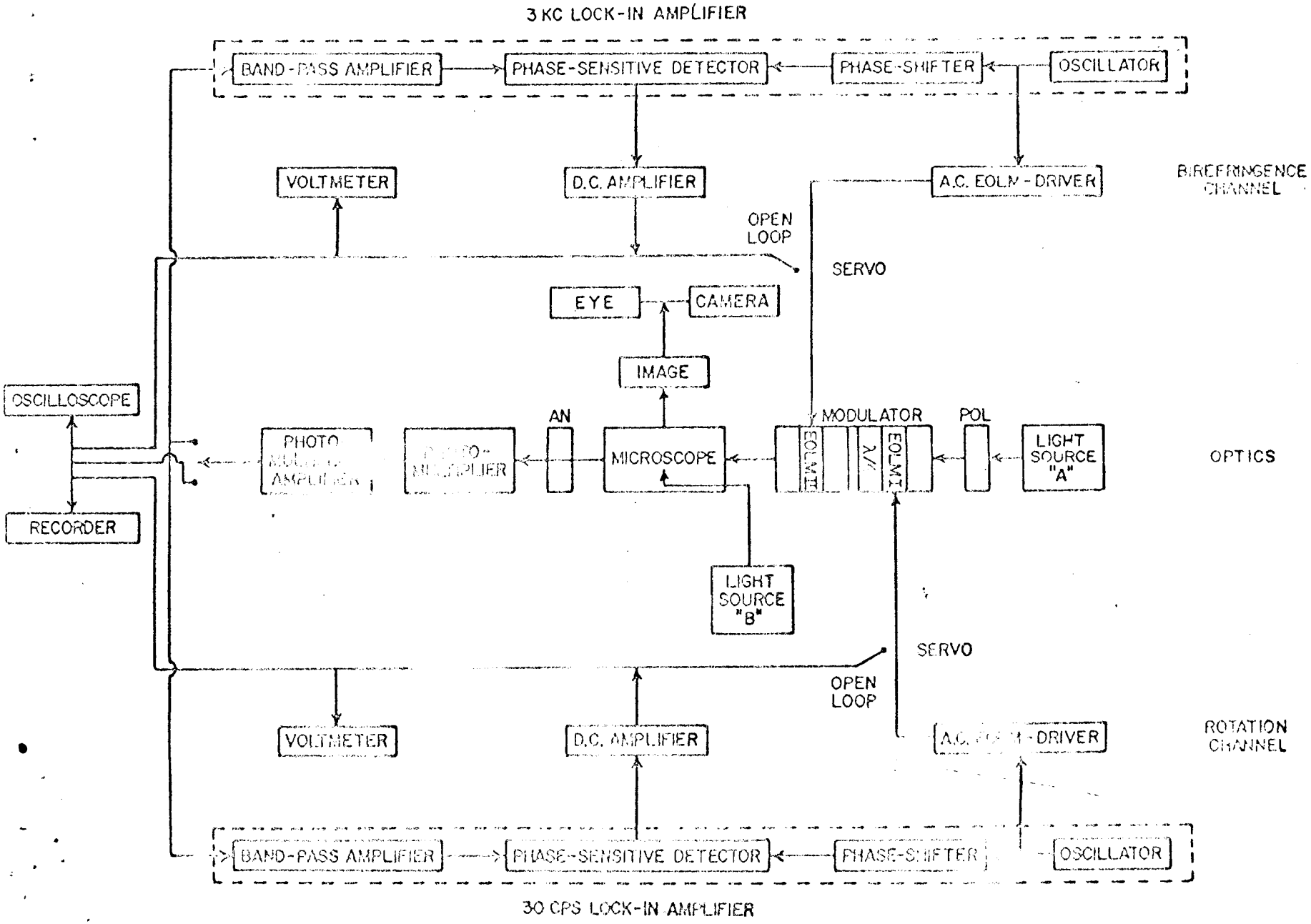
Perhaps the most important application of this system in cell biology will be in interference microscopy, where optical path differences can be used for weighing cells, determining their dry mass concentration, volume, and refractive index. The precision and speed of all interferometric measurements may now be increased by between 10 and 100 times.

light losses due to absorption, that is, microspectrophotometry. The next figure(5) shows schematically six different methods which may be used to detect such losses with varying degrees of precision and specificity for absorption in the face of other possible types of losses present as well, such as refraction and light scattering. The last three methods employ a double-beam. These, like the birefringence measurement method, are limited only by photon noise from the light source. The observer sees two spots in the field, one large and the other small. The latter is the sample beam which detects light losses in a specimen drawn across it; the former is the reference beam, which like the reference beam of an interference microscope must be kept clear of any debris. The optical arrangement uses the retardation of the EOLM to act as a linear servo measuring and compensating for light losses due to absorption. Any part of the transmission scale may be expanded, so that extremely small changes in transmission can be recorded as rapidly as in one millisecond.

The third class of functions of the PAR-ZEISS instrument is what might be called "transmission analogue" measurements of fluorescence and light scattering. Here again a number of methods are possible, but those of choice are double-beam methods using the servo and a comparison standard of either fluorescent material of a known concentration or a light-scattering material. Since in these arrangements, the instrument is essentially a comparison photon counter, it should be possible to measure the concentration of fluochrome, something which would be of great convenience in quantitative studies of the localization of fluorescein-labelled antibodies, etc.

While time has not permitted me to go into the detail of any of these methods, I hope I have driven home the point that the information that may be extracted from any microscopic specimen may be greatly

increased if single optical properties are selectively encoded by modulated light for electrical registration. This represents a new approach and seems to be the method of choice for all kinds of physical microscopic measurements.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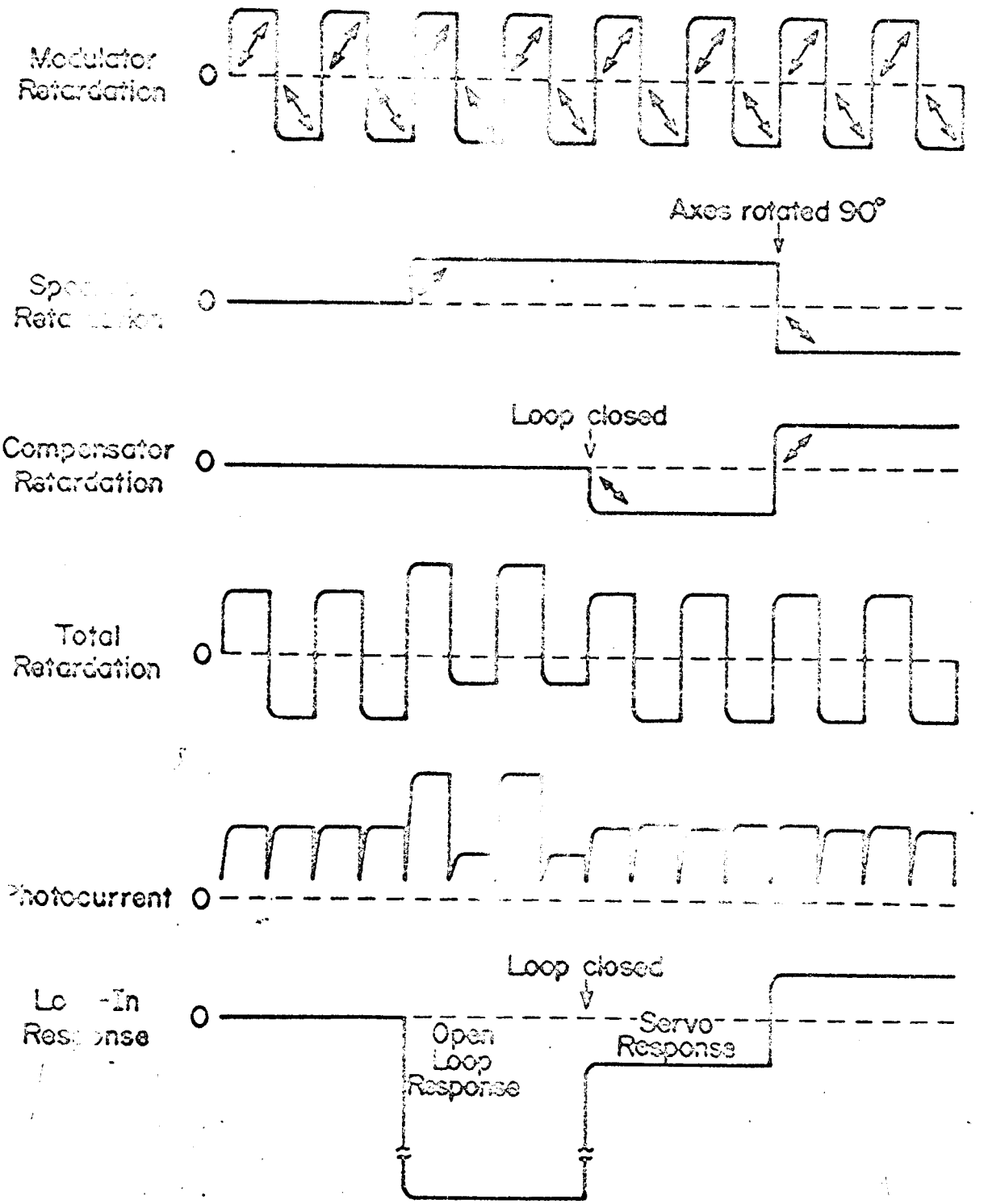
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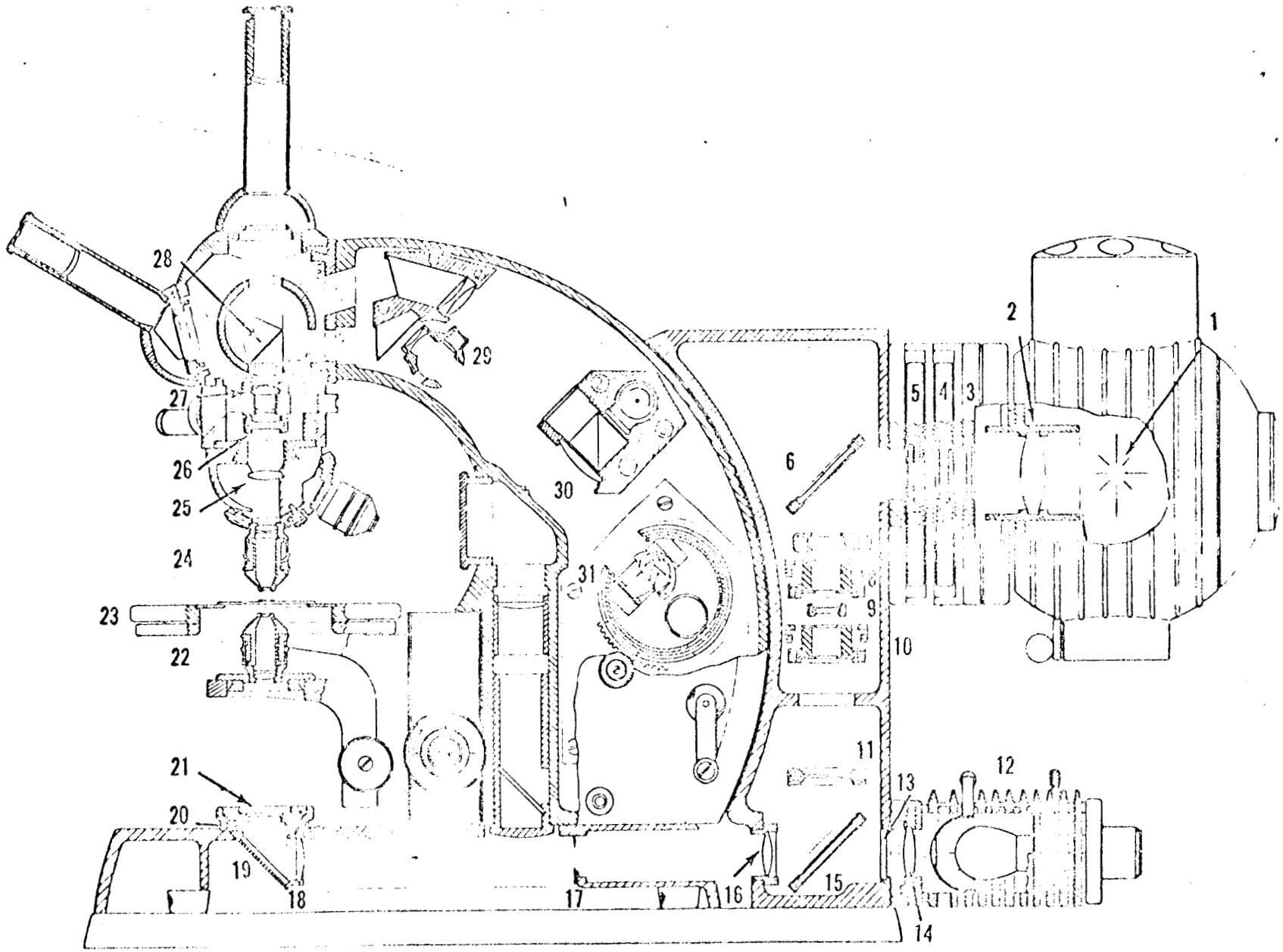
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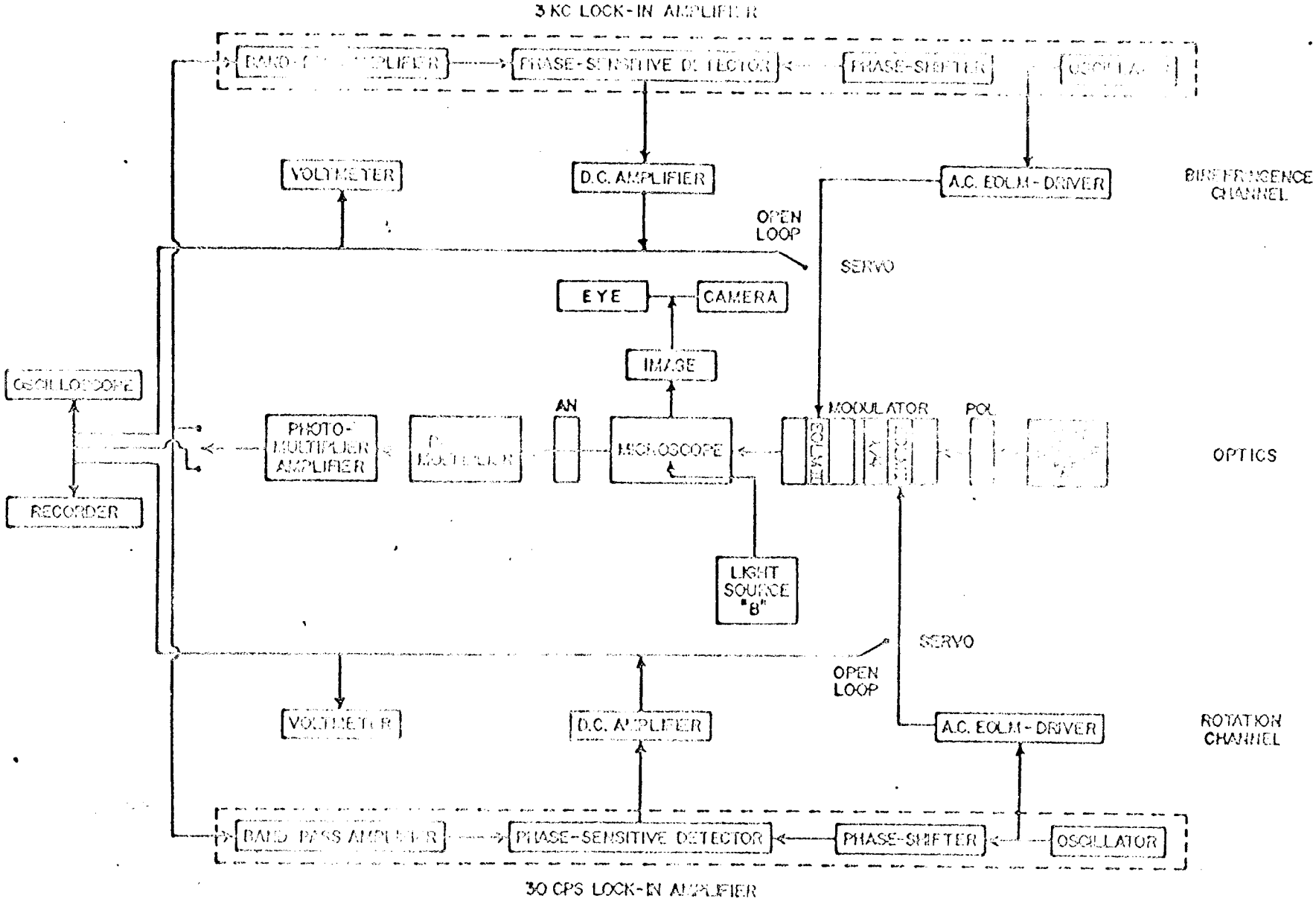
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POLARIZED LIGHT [illegible] INTERFERENCE MICROSCOPY

OPTICAL PROPERTY	MODE	SOURCE	ASSOCIATION	PHOTO-MULTIPLIER	OUTPUT
Ph[illegible] ctor [illegible] e to [illegible] ringe	Servo	[illegible] 0° [illegible]	St[illegible] Optics		Lock-In Amplifier
C[illegible] cal Ro[illegible] Alon[illegible]	Servo	AP, POL, EOLM I, λ/4 (0°, 45°, 0°)	Strain-Free Optics	AN (90°)	Lock-In Amplifier
Optical Rotation in Birefringent Specimens	2 Channels 2 Servos	AP, POL, EOLM I, λ/4, EOLM II (0°, 45°, 0°, 45°)	Strain-Free Optics	AN (90°)	Lock-In Amplifier
Phase Retardations Due to Refraction	Servo	AP, POL, EOLM I (0°, 45°)	Interference Optics	λ/4, AN (0°, 90°) Rotatable	Lock-In Amplifier
Dichroism (Detection Only)	Open Loop	AP, POL, EOLM I, λ/4 (0°, 45°, 0°)	Strain-Free Optics Specimen Rotated		Lock-In Amplifier