TRANSMISSION COATING CAREFULLY CHOSEN

The choice of the balancing inconel transmission coating is therefore a delicate one, and it has been carefully studied. Assuming that the specimen is nearly transparent in bright-field illumination, the determination of the transmission is contained in the Bausch & Lomb objectives. If, however, the specimen absorbs strongly in certain areas, a different balance would have to be established. But, in that case, the specimen absorption in itself produces contrast. The phase accessories increase contrast in the non-absorbing sections of the specimen, yielding a desired contrast balance.

Theoretically, monochromatic light of the same wavelength used for the original calibration, should be used when working with phase accessories if the full phase shift effect is to be realized. It is for this reason that green filters are made available with the Bausch & Lomb Phase Contrast Accessories. The one-quarter wavelength phase shift produced by the B&L accessories, is calculated in the green portion of the spectrum. Use of other wavelengths will not produce the same degree of phase shift. If the microscopist chooses to use white light, extinction by interference will not be complete for all colors, since the specimen detail does not exert the same phase altering power for all colors.

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PHASE CONTRAST MICROSCOPY

Unstained, colorless, transparent specimens under standard bright field illumination are generally lacking in contrast. In order to obtain contrast so that structural detail may be recognized and studied, time consuming methods of preparation have been necessary. These have often resulted in physical or chemical changes in the specimen, especially in the case of living biological material.

The phase contrast method of microscopy overcomes this difficulty. It permits the examination of living, unstained material without resort to the usual staining procedure. The method is also applicable to fixed and dry specimens including microtome sections. Provided mounting media of correct index are employed, selective differentiation of structure in fixed specimens is often possible without the use of stains.

BAUSCH & LOMB INCORPORATED
ROCHESTER, NEW YORK, U. S. A.

KENNETH A. DAWSON CO.
106 CONCORD AVE.
BELMONT 78, MASS.

IN BRIEF...

On these pages is a detailed explanation of the principle of phase contrast. To readers interested in the mechanics of this technique it will prove very illuminating. For others, primarily concerned with the end result, a condensed explanation is more in order.

Phase Contrast Microscopy is, essentially, a special method of controlled illumination, ideally suited for observing thin, transparent objects whose structural details vary only slightly in thickness and refractive index.

These slight variations are not sufficient in themselves to be visible in the bright-field microscope. They do, however, affect light passing through the specimen, causing slight irregularities in the wave fronts. Minor changes in the phase relation of the wave fronts result. Phase contrast equipment transforms these phase changes into corresponding variations of brightness, so that the structure is visible under conditions of enhanced contrast.

RESOLVING POWER OF THE MICROSCOPE

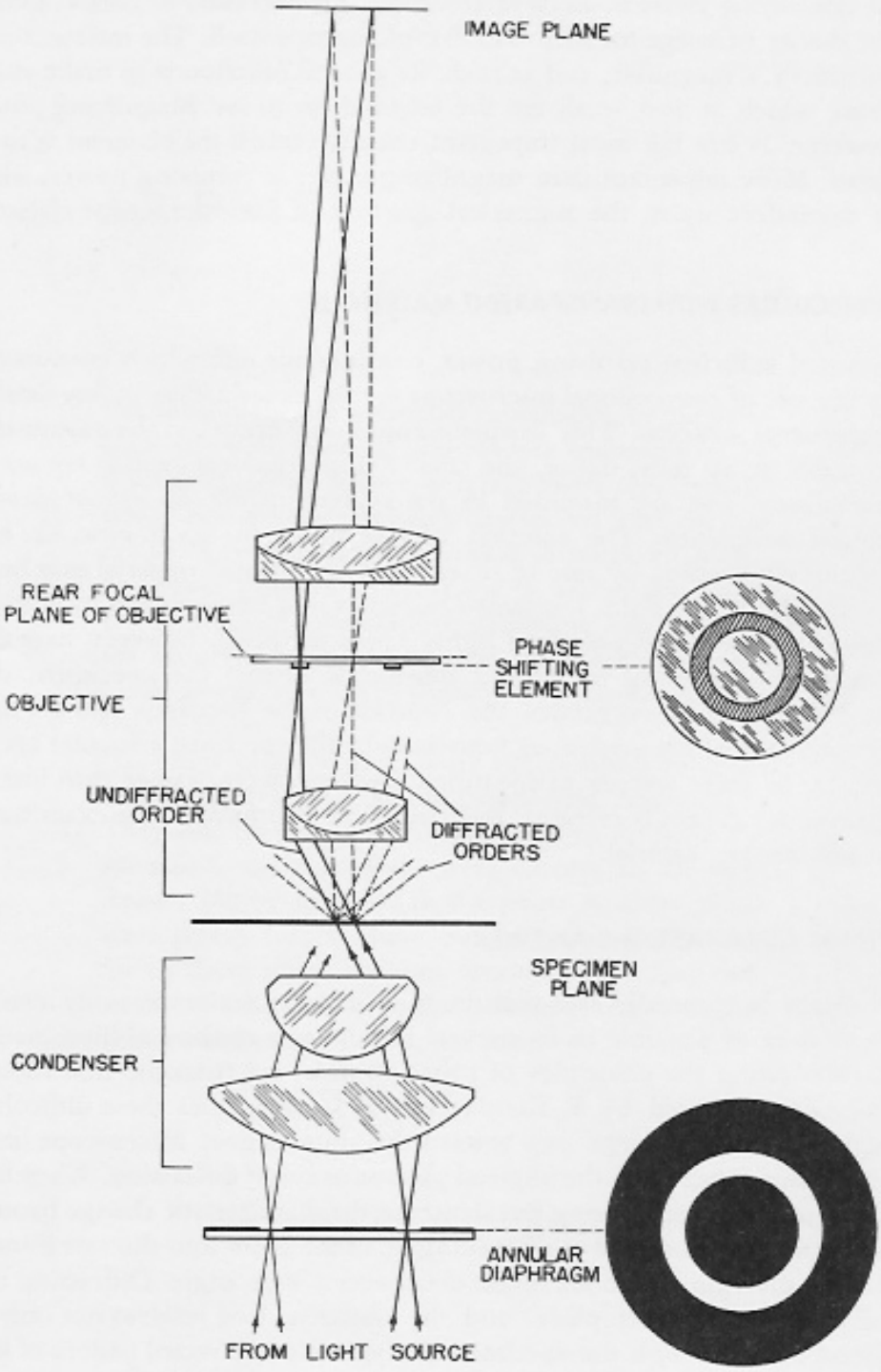
In describing phase contrast microscopy, it is necessary to review, briefly, the theory of image formation in the microscope itself. The microscope is essentially a magnifier, and as such, its general function is to make visible detail which is too small for the unaided eye to see. Magnifying power, however, is not the most important characteristic if the observer is to see detail. More important than magnifying power is resolving power, which is dependent upon the numerical aperture of the microscope objective.

DIFFICULTIES WITH TRANSPARENT MATERIALS

Granted sufficient resolving power, considerable difficulty is encountered in the use of conventional microscopy for the examination of fine detail in transparent material. This problem commonly occurs in the examination of many living cells, tissue, and small living organisms which are usually transparent and are mounted in transparent media of almost identical optical properties. The solution of this problem, up to now, has been commonly reached by one of three methods. Stained material may be examined by bright field. Unstained materials may be examined by either or both dark field and polarized light. These methods, however, have their limitations. Staining techniques oftentimes distort the specimen; dark field sometimes exaggerates the contrast of the specimen and the background so that the gradations possessed by the specimen structure are not shown in their proper proportions. Surface layers, rather than internal details, are generally revealed. Polarized light is limited to the examination of anisotropic material.

PHASE CONTRAST, THE ANSWER

It might be generally said that the microscopist desires to study material in as near as possible to its natural state. A new method of illumination, incorporating the principles of phase contrast microscopy, first successfully demonstrated by F. Zernike in 1935, overcomes these difficulties, and is at least a large step toward the ultimate goal. Microscope image formation depends on the physical phenomenon of diffraction. When light falls upon an object having fine structure, the characteristic change brought about may be described as a breaking up of the beam into discrete bundles of rays diverging from the object detail over a wide angle. Diffraction thus occurs at the object plane, and the objective lens receives not only the direct light through the specimen but all other diffracted orders of light falling within the angular limits of the objective aperture. If the source is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PHASE SHIFTING ELEMENT
UNDIFFRACTED ORDER
DIFFRACTED ORDERS
SPECIMEN PLANE
CONDENSER
ANNULAR DIAPHRAGM
FROM LIGHT SOURCE

one providing parallel light, all such bundles entering the objective are imaged at its rear focal plane where they may be observed as a series of maxima and minima diffraction spectra images. In this plane, these images act as secondary sources, and send out their own cones of light. Since these images have related phases in this plane, their effect in any other plane may be influenced, controlled and measured through observable effects. Hence, since all of the light emanates from the same original source, the related spectra become visible through their interference effects wherever they overlap.

It is at this point, in the final image plane, that conditions of structure and phase, as they exist in the original specimen, are reproduced. If a change is introduced in the relative phases of the spectra appearing at the rear focal plane of the objective, the intensity in the final image will vary. Evidence of structure detail will be revealed as a consequence. In the rear focal plane of the objective, a phase difference is introduced in the phase contrast method of microscopy. In this manner, it is possible to suppress, intensify, and even reverse the contrast in the final image, depending upon the nature of the phase shift introduced.

PHASE ALTERING ANNULI

Phase altering patterns, commonly referred to as "phase plates," are expedients designed to effect a change in an optical path, or phase relation, between that part of the light entering the objective directly through the object structure, and that part which is diffracted by the structure and also enters the objective. Such phase altering patterns are made by the deposition of films of predetermined thicknesses by high vacuum thermal evaporation processes. These films may be applied to separate glass discs mounted in the objective, or applied directly to a lens surface, depending upon the

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IMAGE FORMATION, PHASE CONTRAST

An annular aperture in the diaphragm, placed in the focal plane of the substage condenser, controls the illumination on the object. The aperture is imaged by the condenser and objective at the rear focal plane, or exit pupil, of the objective. In this plane a phase shifting element, or phase plate, is placed.

Light, shown by the solid lines and undeviated by the object structure, in passing through the phase altering pattern, acquires a one-quarter wavelength of green light advance over that diffracted by the object structure (broken lines) and passing through that region of the phase plate not covered by the altering pattern. The resultant interference effects of the two portions of light form the final image. Altered phase relations in the illuminating rays, induced by otherwise invisible elements in the specimen, are translated into brightness differences by the phase altering plate.
(The eyepiece is not shown in this diagram.)

objective design. Patterns, which have been found to be most practical and effective, are those of the annular type, which introduce a phase shift of one-quarter of a wave-length of green light.

An annular aperture diaphragm is necessary for all types of annular phase altering patterns. It is placed at the front focal plane of the substage condenser and, when illuminated, becomes an effective source at infinity with respect to the object plane. Such an annular source illuminates the object with a hollow cone of light, and controls the obliquity of the light traversing the medium.

The shape of the illuminating aperture is important in the formation of diffraction images, since such images actually determine the nature of the final image through their interference effects, and are images not of the specimen itself, but of the illuminating aperture as influenced by the specimen structure.

For the most critical conditions of illumination, light is focused on the substage condenser annular diaphragm, using the principles of Koehler illumination, to fill it uniformly with light. The real image of the diaphragm is then reproduced through the condenser, object, and objective, at the rear focal plane of the objective.

This, then, satisfies the condition that the object may be considered to be illuminated by a number of point sources located at infinity. Also, at the rear focal plane are imaged the diffraction spectra produced by the object structure.

FORMATION OF PHASE DIFFERENCE

The phase altering annulus is placed in the rear focal plane of the objective and its size is such that it coincides with the image of the substage annulus. When the two annuli are exactly concentric and superimposed, direct transmitted light will pass through the annular region and will differ in phase by one-quarter of a wavelength from the light diffracted by the object and entering the objective. This diffracted light passes through the remaining aperture area, not covered by the annulus.

Since the effect of the shift may be either to accelerate or retard the phase relation of the directly transmitted light, with respect to the diffracted portion, either positive or negative contrast may result. If the film thickness contributing to the phase shift is such that the directly transmitted light passing through the phase altering element is effectively accelerated over the diffracted light by one-quarter of a wavelength of light, the resulting contrast is referred to as "positive" or "dark" contrast, and the phase altering element is said to be "accelerating." Positive contrast shows regions of

greater optical path in the specimen to be darker than the surrounding background.

"Negative" or "bright" contrast is produced by a retardation of the undiffracted light, regions of greater optical path in the specimen appearing bright against a darker background. The image is thus similar to a dark field image. While such an objective is easily produced, popular demand has been in favor of "dark" contrast, since the results obtained have greater similarity to the usual hematoxylin stained bright field image. An added benefit is greatly enhanced contrast and graded variations.

Since most microscopic specimens exhibit irregular structure, the diffraction spectra are not clearly defined, and the direct image of the illuminating source, only, is sharply defined. This direct light, in addition, is much more intense than the diffracted light.

METALLIC ABSORBING FILM

Since the direct light always completely participates in the image formation, its intensity would tend to overbalance that from the weaker, secondary diffraction maxima. The relative distribution of light between these two determines the amount of contrast available. To compensate for this unbalanced distribution of light, a metallic absorbing film is combined with the phase element to reduce the intensity of the direct light. This absorption tends to equalize the intensities of the two interfering beams and thus increases the sensitivity of contrast by permitting the weak diffracted orders to effect a proportionately greater influence.

If the direct beam is not sufficiently dampened (inconel deposit on the phase annulus) to equalize its brightness with that of the diffracted beam, the two will interfere, but there will be an excess of light in the direct beam beyond that necessary for the interference process. That direct light will dilute image contrast. If it is excessive, regular bright field microscopy results. The diffracted beam would be drowned out. Similarly, if the diffracted beam is out of balance, the image will be devoid of contrast.

The latter results if the inconel deposit be made opaque and the diffracted beam, only, proceeds to the image plane. It is, therefore, obvious that by varying the transmission of the annulus, there can be produced, at will, the complete range from no contrast all the way to a pseudo dark field. The best contrast somewhere in between is that giving a gradation of darkness for the various degrees of densities within the specimen. Bright field gives no contrast at all unless the specimen is thick or absorbs strongly, and dark field gives the extreme black and white contrast occluding everything in between.