# Contrast Methods in Microscopy
## Transmitted Light

By Mortimer Abramowitz
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For Olympus Corporation

Volume 2

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## Transmitted Light

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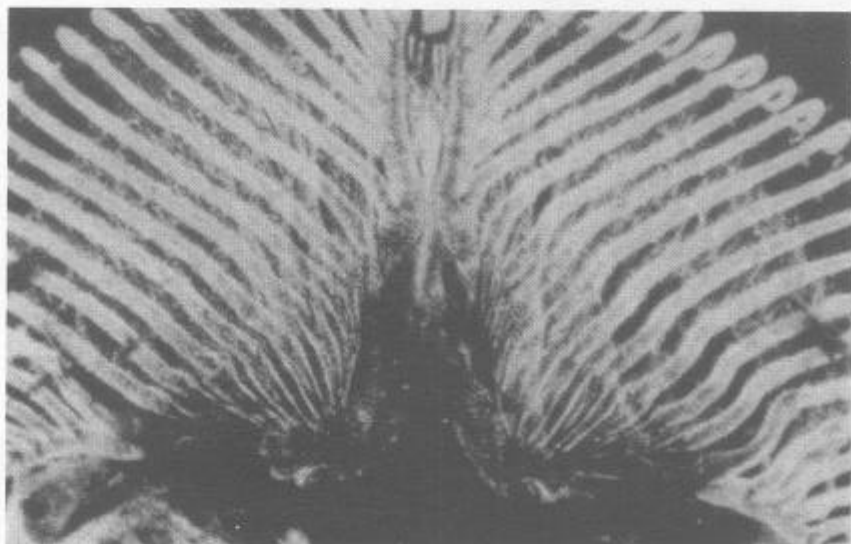


Bottom: Embryonic
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Brightfield
Illumination

## Introduction

In Volume 1 of this series (Microscope Basics and Beyond), the principles of transmitted light brightfield microscopy were explained and illustrated. For purposes of introduction, image formation was 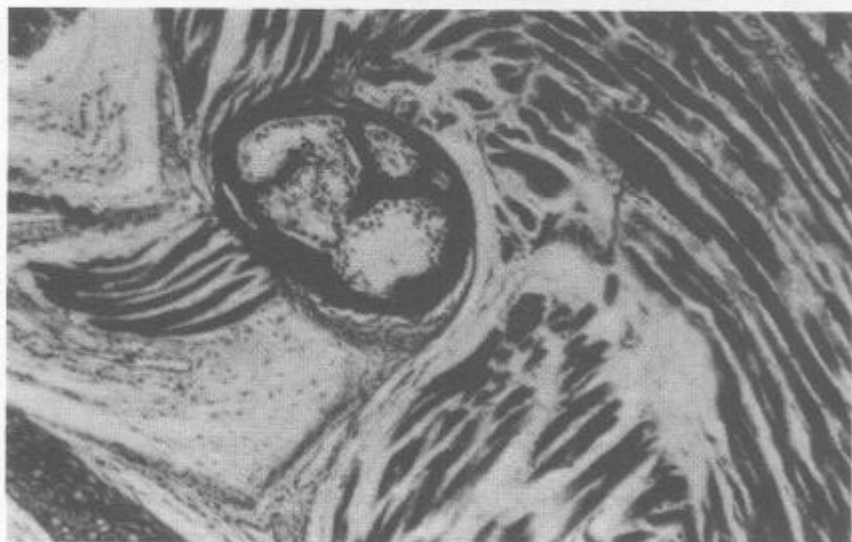described mostly in terms of geometric optics* with only brief reference to the wave action of light and the phenomenon of diffraction.**

Volume 2 will give a more extended treatment of image formation as related to diffraction (wave optics or physical optics). Such explanations are crucial to the understanding of all image formation and indispensable for understanding phase microscopy, differential interference microscopy, and polarization. This booklet will present an overview of principles and their applications in the most utilized contrast methodologies devised to improve image contrast and visibility. The emphasis will be on practical use and basic ideas with a minimum of mathematics. Bear in mind that when you look through the eyepiece of a microscope, you are looking at the image projected by the objective onto the plane of the fixed diaphragm inside the eyepiece. In order to see that image, it must be enlarged enough to fall on separate cells (rods and cones) on your retina (magnification); the details of the image must be separated sufficiently so as to be clearly distinguishable (resolution) and the image of the object must vary in brightness from the background (contrast) so that it is visible. Remember that when you look into the microscope, you are not looking at the specimen; you are looking at a magnified *image* of the specimen by means of the eyepiece's eye lens. (Fig. 1)

*Fig. 1. Compound Microscope-Schematic.*



EYEPIECE

PLANE OF INTERMEDIATE IMAGE

BACK FOCAL PLANE OF OBJECTIVE

OBJECTIVE

SPECIMEN

CONDENSER

APERTURE DIAPHRAGM
FRONT FOCAL PLANE OF CONDENSER

FIELD DIAPHRAGM
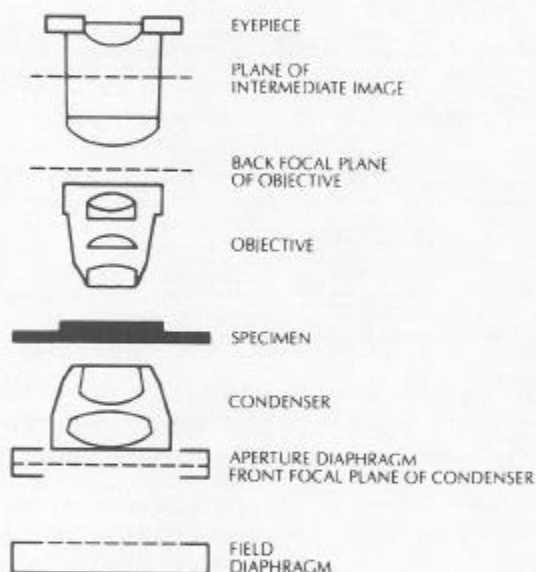
*Fig. 1*

The human eye can readily distinguish light and dark (variations in brightness or intensity) as well as colors (light frequencies) of the visible part of the spectrum. A microscope image is essentially a pattern of varying light intensities and colors.

In microscopy, some specimens, so-called amplitude objects because they absorb light partially or completely, are readily seen. Specimens that are naturally colored or artificially stained with chemical color dyes can also be seen. These stains or natural

*Geometric optics treats light as rays traveling in straight lines. Directions are altered by reflection or refraction by lenses. The structure of the object is not taken into account as it is in wave optics.

**Explanations of diffraction will be dealt with later.

colors absorb some part of white light passing through and transmit or reflect other colors. As a result, the object appears in one or several colors. Often, stains are combined to yield contrasting colors, e.g. blue haemotoxylin stain for cell nuclei combined with pink eosin stain for cytoplasm. It is common practice to use stains on specimens that do not absorb light, thus rendering such objects visible to the eye.

## CONTRAST

Thus, absorption of light, brightness or color, has been the classical means of producing contrast in microscope images. Contrast means the ability of a detail to stand out against the background or *other adjacent details*. Contrast refers to the relationship between the highest and lowest intensity in the image. In terms of a simple formula, contrast is described as:

$$\% \text{ Contrast} = \frac{\text{Intens. of background} - \text{Intens. of specimen}}{\text{Intens. of background}} \times 100$$

For many specimens in microscopy, especially unstained or living material, the contrast is so poor that, despite the ability of the objective to resolve (clearly separate) the details, the object remains virtually invisible. (Page 12) Often, for just such specimens, it is important not to alter them by killing or treatment with chemical dyes or fixatives. Thus, for over a hundred years, microscopists have sought ways of increasing contrast in unstained material without doing chemical or thermal "violence" to the object being studied. The all-too-common practices of closing down the condenser aperture diaphragm below 60% or lowering the substage condenser do indeed increase contrast but such procedures very seriously reduce resolution and sharpness.

## BACK FOCAL PLANE OF THE OBJECTIVE

In order to understand many of the methods of contrast improvement, you must be familiar with the optical phenomena that are taking place, especially the optical "action" at the back focal plane of the objective. You can readily observe such phenomena by looking at the back lens area of the objective (first, removing the regular eyepiece) with a phase telescope. This relatively inexpensive device is really a low power magnifier which can easily be focused on the back focal plane of the objective.

## WAVE NATURE OF LIGHT

Some brief non-technical description concerning the wave nature of light: Light is considered as a disturbance moving outward in all directions from the origin of the disturbance, e.g., a bulb filament. Compare light with a disturbance caused by dropping a rock into a still pond. The disturbance moves out in all directions; consider this motion as the *propagation* directions of the disturbance. As this disturbance moves outward, waves are created which vibrate up and down, PERPENDICULAR to the directions of propagation. (Fig. 2) If the original energy source (the dropped rock) is great, the waves are higher; conversely, if the energy source is lesser, the waves are lower in height.

The height of each wave is called the amplitude of the wave. If you were to measure from the crest of one wave to the crest of the adjoining wave, you would find the wave length of the wave (measurement from trough to trough, or any comparable pair of positions would also yield the same wave length). (Fig. 3) In the case of light, the speed of propagation is the familiar 186,000 + miles per second in a vacuum. The number of vibrations per second is known as the frequency and is invariant for each of the colors of white light.
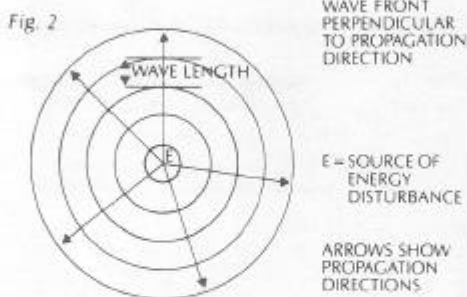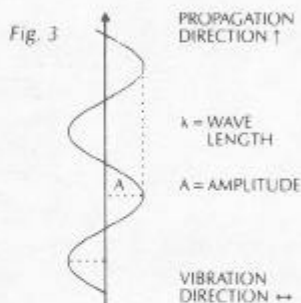
Fig. 2. Wave Formation

Fig. 3. Wave Diagram— Schematic

Fig. 2

WAVE LENGTH

WAVE FRONT PERPENDICULAR TO PROPAGATION DIRECTION

E = SOURCE OF ENERGY DISTURBANCE

ARROWS SHOW PROPAGATION DIRECTIONS

Fig. 3

PROPAGATION DIRECTION ↑

$\lambda$ = WAVE LENGTH

A = AMPLITUDE

VIBRATION DIRECTION ↔

## REFRACTIVE INDEX

When light enters another medium from air, e.g. glass, the light waves are slowed down and the wave length of any given wave frequency is compressed. Upon reemergence into air, light resumes its former speed and the wave length is no longer compressed. (Fig. 4) For practical purposes, you can consider the speed of light to be
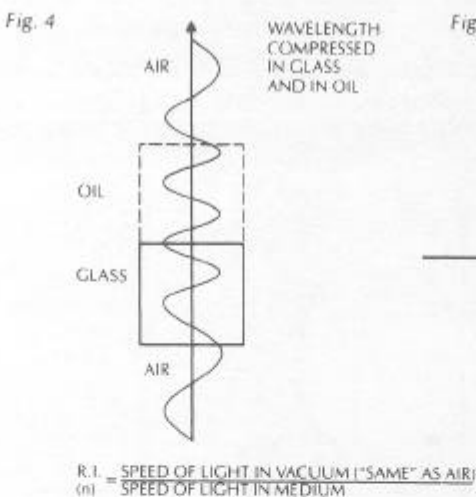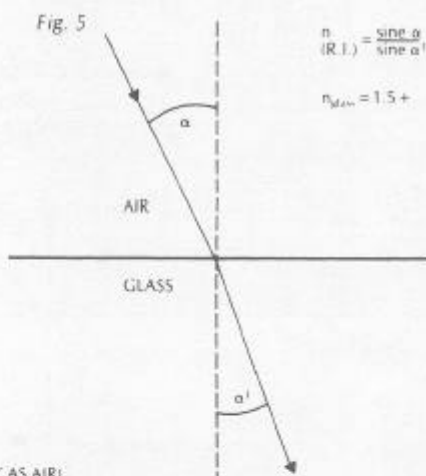
Fig. 4. Refractive Index

Fig. 5. Snell's Law

Fig. 4

AIR

WAVELENGTH COMPRESSED IN GLASS AND IN OIL

OIL

GLASS

AIR

$$\frac{R.I.}{(n)} = \frac{\text{SPEED OF LIGHT IN VACUUM ("SAME" AS AIR)}}{\text{SPEED OF LIGHT IN MEDIUM}}$$

Fig. 5

AIR

GLASS

$$\frac{n}{(R.I.)} = \frac{\text{sine } \alpha}{\text{sine } \alpha'}$$

$$n_{glass} = 1.5 +$$

the same in air as it is in a vacuum. However, light is considerably slowed when it enters water or oil or glass. The ratio of the speed of light in air (or in a vacuum) as compared to the speed of light in another medium is known as the refractive index or R.I. (or n). Another way of describing R.I. is the ratio of the sine of the angle of light entering the medium from air (angle of incidence) compared to the sine of the angle of light after it has entered the medium. (Fig. 5) Since glass, oil, water etc. slow light down, their refractive indices are always greater than 1. (The speed of light in air is considered to be 1.)
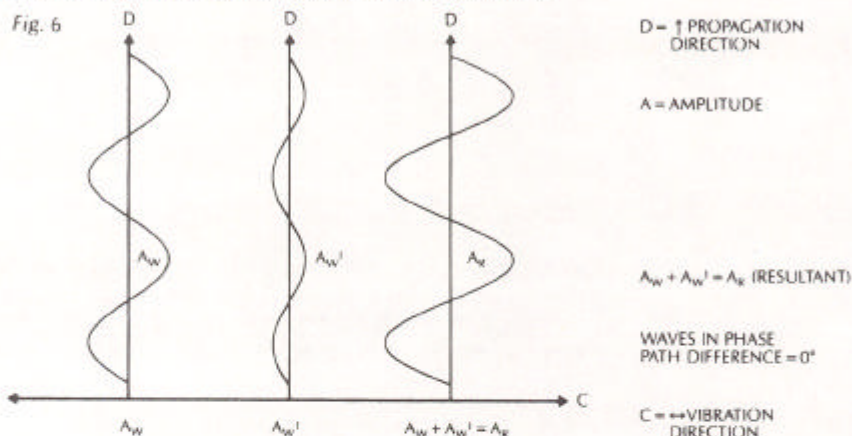
# CONSTRUCTIVE AND DESTRUCTIVE INTERFERENCE

An important characteristic of light waves is their ability, under certain circumstances, to interfere with one another. If a pair of light waves from the same source are traveling, for example, in direction D (propagation direction) and their vibrations (perpendicular to the propagation direction) are parallel to each other and parallel to C, the light waves may interfere with each other. If the vibrations are not in the same plane and are vibrating at 90 degrees to each other, they cannot interfere with one another.

Assuming the vibrations are parallel to one another and to C:

If the crests of one of the waves coincide with the crests of the other, the amplitudes are additive. If the amplitudes of both waves are equal, the resultant amplitude would be doubled. Bear in mind that light intensity varies directly as the SQUARE of the amplitude. Thus if amplitude is doubled, intensity is quadrupled. Such additive interference is called *constructive interference*. (Fig. 6)

*Fig. 6. Constructive Interference*

Fig. 6

D = ↑ PROPAGATION DIRECTION

A = AMPLITUDE

$A_W + A_W' = A_R$ (RESULTANT)

WAVES IN PHASE
PATH DIFFERENCE = 0°

C = ↔ VIBRATION DIRECTION

If the crests of one wave coincide with the troughs of the other wave, the resultant amplitude is decreased or may even be completely canceled. (Fig. 7) This is called *destructive interference*. The result is a drop in intensity, or, in cancellation, blackness.

*Fig. 7. Destructive Interference*

Fig. 7

D = ↑ PROPAGATION DIRECTION

A = AMPLITUDE

$A_W - A_W' = A_R$ (RESULTANT)

C = ↔ VIBRATION DIRECTION
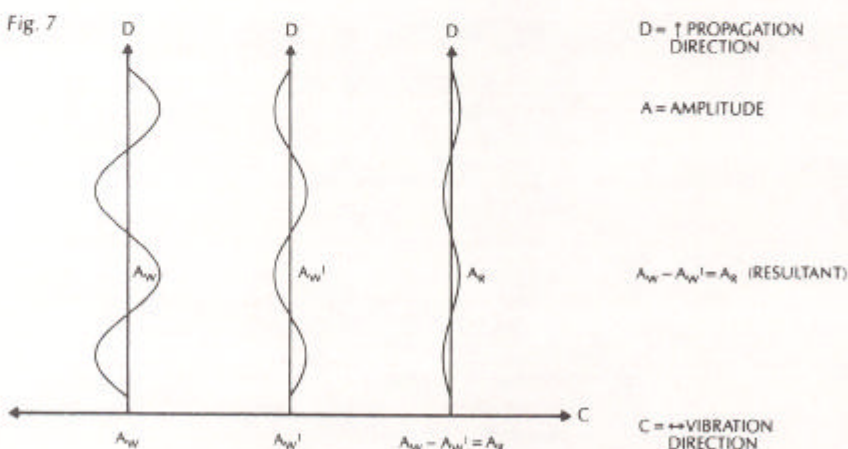
# UNDEVIATED AND DEVIATED LIGHT

In the light microscope, where light from the microscope bulb passes through the condenser and then through the specimen—*assuming the specimen is a light absorbing*

specimen—some of the light passes through the specimen or around the specimen undisturbed in its path. Such light is called direct light or undeviated light. The background light (called the surround) around the specimen is also undeviated light. On the other hand, some of the light passing through the specimen is deviated. (Fig.8) Such deviated light (as you will subsequently learn, called diffracted light) is rendered ½ wave length or 180 degrees out of step (out of phase) with the direct light that has passed through undeviated.

*Fig. 8. Light Deviated (Diffracted) Passing through a Narrow Opening of Specimen— Schematic*

*Fig. 8*
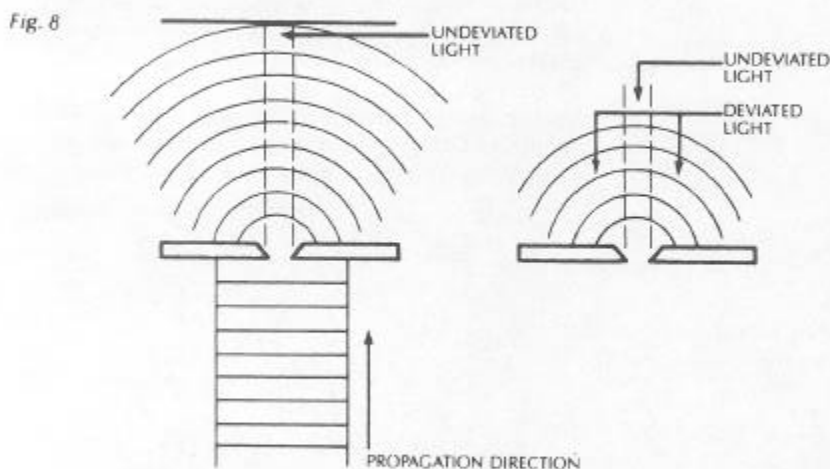


## THE IMAGE

The half wave length out of step caused by the specimen itself enables this light to cause destructive interference with the direct light when both arrive at the IMAGE plane at the diaphragm of the eyepiece. The eye lens of the eyepiece further magnifies this image which finally is projected onto the retina or the camera film.

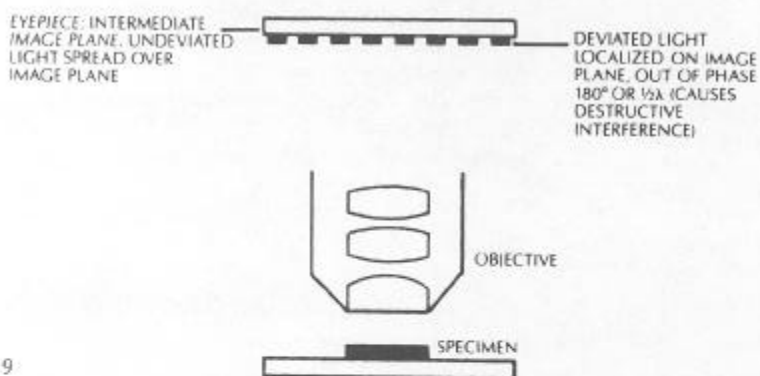*Fig. 9 Schematic Image formation— Absorbing Specimen*



*Fig. 9*

What has happened is that the direct or undeviated light is projected by the objective and spread evenly across the entire IMAGE plane at the diaphragm of the eyepiece. The light diffracted by the specimen is brought to focus at various *localized places* on that same IMAGE plane; and there the diffracted light causes destructive interference, and reduces intensity resulting in more or less dark areas. (Fig. 9) These patterns of light and dark are what we recognize as an image of the specimen. Since our eyes are sensitive to variations in brightness, the image then becomes a more or less faithful reconstitution of the original specimen.

# IMAGE FORMATION RESULTING FROM DEVIATED AND UNDEVIATED LIGHT

To help you understand the basic principles, it is suggested that you try the following exercise and use as your "specimen" an object of known structure, e.g. a stage micrometer or a grating of closely spaced dark lines. Place such a finely ruled grating on the microscope stage and bring it into focus using first a 10X and then the 40X objective. Remove the eyepiece and, in its place, insert a phase telescope so that you can focus on the BACK focal plane of the objective. If you close down the condenser diaphragm most of the way, you will see a bright white central spot of light which is the image of the aperture diaphragm. To the right and left of the central bright spot, you will see a series of spectra, each colored blue on the part closest to the central spot and colored red on the part of the spectrum farther from the central bright spot. (Fig. 9A) The intensity of these colored spectra decreases according to how far the spectrum is

Fig. 9A

Intermediate Image—

EYEPIECE DIAPHRAGM

DIFFRACTION PATTERN AT BACK FOCAL PLANE OF OBJECTIVE
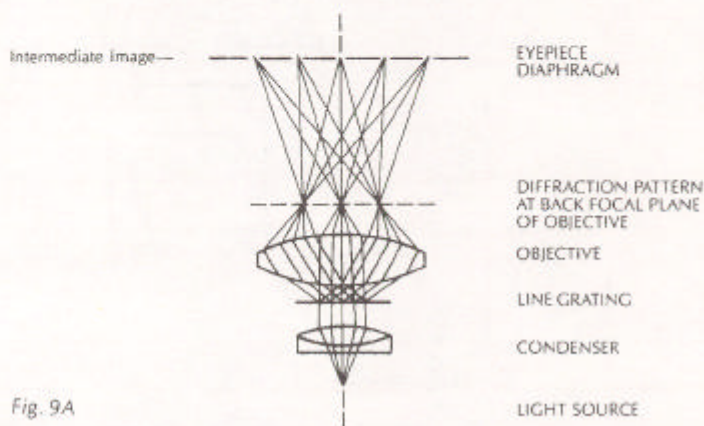
OBJECTIVE

LINE GRATING

CONDENSER

LIGHT SOURCE

from the central spot. Those spectra nearer the periphery of the objective are dimmer than those closer to the central spot. If you remove the grating from the stage, these spectra disappear and only the central image of the aperture diaphragm remains. If you put back the grating, the spectra reappear. Note that the spaces between the colored spectra appear dark. If you examine the grating with the 10X objective, you will observe that only one pair of spectra can be seen, one to the left of the central spot, one to the right. If you examine the line grating with a 60X objective (assuming it has a higher numerical aperture than your 40X), you will observe more spectra to the right and left than you were able to see with the 40X in place. (Fig. 10 A-D)

Fig. 10



| 40X OBJECTIVE— LINE GRATING REMOVED | 60X OBJECTIVE, N.A. 0.85 | 10X OBJECTIVE, N.A. 0.25 | 40X OBJECTIVE, N.A. 0.65 |

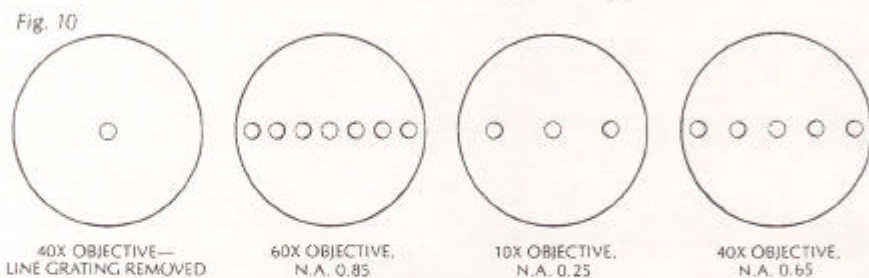Since the colored spectra disappear when the grating is removed, it can be assumed that it is the specimen itself which is affecting the light passing through, thus producing the colored spectra. Further, if you close down the aperture diaphragm, you will observe that objectives of higher numerical aperture "grasp" more of these colored spectra than do objectives of lower numerical aperture. The crucial importance of

these two statements for understanding image formation will become clear in the ensuing paragraphs.

## THE DIFFRACTION PATTERN AND IMAGE FORMATION

The central spot of light (image of the condenser aperture diaphragm) represents the direct or undeviated light passing through the specimen or around the specimen undisturbed. It is called the 0th or zeroth order. The fainter colored images of the aperture diaphragm on each side of the zeroth order are called the 1st, 2nd, 3rd, 4th etc. orders respectively. All the "captured" orders represent, in this case, the *diffraction pattern* of the line grating as seen at the back focal plane of the objective. (Fig. 11)

Fig. 11
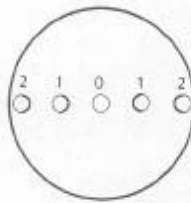
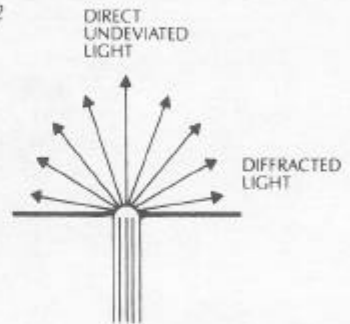Fig. 12



DIFFRACTION PATTERN
OF LINE GRATING

DIRECT
UNDEVIATED
LIGHT

DIFFRACTED
LIGHT

The fainter colored diffracted images of the aperture diaphragm are caused by light deviated or diffracted, spread out in fan shape, at each of the openings of the line grating. (Fig. 12) The blue wave lengths are diffracted at a lesser angle than the green wave lengths which are at a lesser angle than the red wave lengths.

At the back focal plane of the objective, the *blue* wave lengths from each slit interfere constructively to produce the blue area of the *diffracted* image of each spectrum or order; similarly for the red and green areas. Where the diffracted wave lengths are ½ wave out of step for each of these colors, the waves destructively interfere. Hence the dark areas between the spectra or orders.

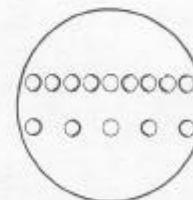At the position of the *zeroth* order, *all* wave lengths from each slit add constructively; this produces the bright white light you see as the zeroth order at the center of the back focal plane of the objective.

The closer the spacings of a line grating, the fewer the spectra that will be "captured" by a given objective. (Fig. 13)

Fig. 13
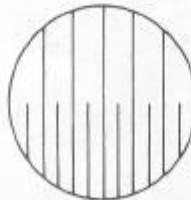


WIDER AND NARROWER SLITS

DIFFRACTION PATTERNS AT BACK
FOCAL PLANE OF OBJECTIVE

The direct light and the light from the diffracted orders continue on, being focused by the objective, to the IMAGE plane at the diaphragm of the eyepiece. (Fig. 14) Here the direct and diffracted light interfere and are thus reconstituted into the real, inverted image that is "seen" by the eye lens of the eyepiece and further magnified.

Fig. 14

A. SPECIMEN    A'. DIFFRACTION PATTERN    B. SPECIMEN    B'. DIFFRACTION PATTERN
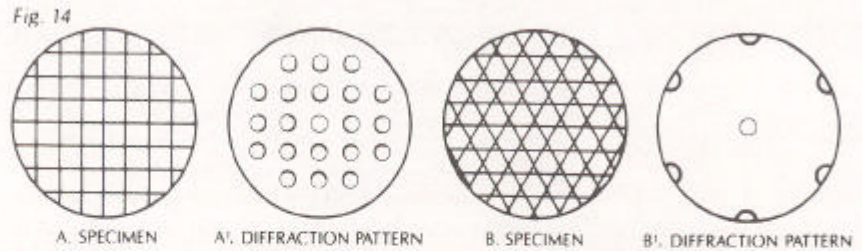
Microscope specimens can be considered as complex gratings with details and openings of various sizes. This concept of image formation was largely developed by Ernst Abbe, the famous German microscopist and optics theoretician of the 19th century. According to Abbe (his theories are widely accepted at the present time), the details of a specimen will be resolved, if the objective "captures" the 0th order of light and at least the 1st order too; or any two orders. *The greater the number of diffracted orders that gain admittance to the objective, the more accurately the image will represent the original object.*

## EFFECT OF OIL IMMERSION AND SHORTER WAVE LENGTH OF LIGHT

Further, if a medium of higher refractive index than air (e.g. immersion oil) is used in the space between the front lens of the objective and the top of the cover slip, the angle of the diffracted orders is reduced, the fans of diffracted light are compressed; as a result, an oil immersion objective can "capture" more diffracted orders and yield better resolution than a dry objective. (Fig. 15 A,B,C) Moreover, since blue light is
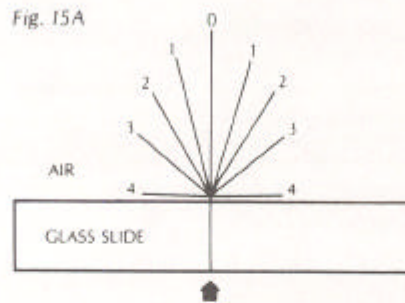
Fig. 15A

0
1  1
2  2
3  3
AIR
4  4

GLASS SLIDE

Fig. 15B

0
1  1
2  2
3  3
4  4
5  5
6  6

GLASS SLIDE

Fig. 15C

40X DRY OBJECTIVE
N.A. 0.85

40X OIL IMMERSION
N.A. 1.0

diffracted at a lesser angle than green light than red light, a lens of a given aperture may capture more orders of light when the light is blue. (Fig. 16)

Fig. 16

60X 0.85 OBJECTIVE



BLUE LIGHT
INCLUDES 0TH, 1, 2, 3, ORDERS

RED LIGHT
INCLUDES 0TH, 1, 2 ORDERS
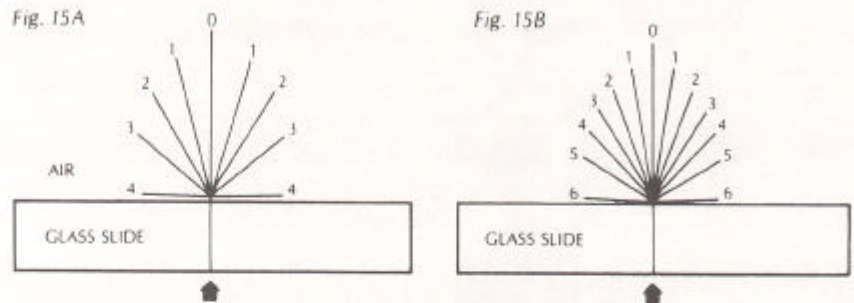
*Fig. 16. Blue Light vs. Red Light Schematic Diffracted Orders Line Grating*
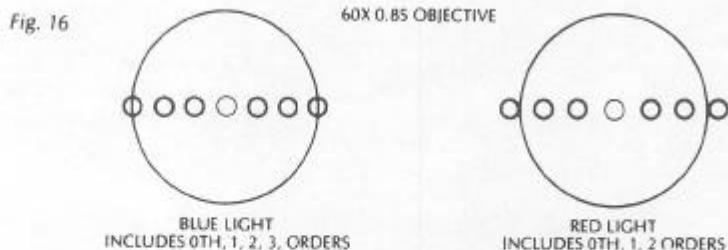
These two principles explain the classic equation often cited for resolution:

$$d = \frac{1.22\lambda}{2N.A.}$$

"d" is the space between two adjacent particles, still allowing the particles to be perceived as separate; $\lambda$ is wave length; N.A. is the numerical aperture of the objective.

The more of the higher diffracted orders admitted to the objective, the smaller the details of the specimen that can be clearly separated (resolved). Hence the value of high numerical aperture for such specimens. Likewise, the shorter the wave length of visible light used, the better the resolution. These ideas explain why high numerical aperture, apochromatic lenses can separate extremely small details in blue light.

## APERTURE DIAPHRAGM AND DIFFRACTED ORDERS

If you were to block out (Abbe did just such experiments) the outermost diffracted orders by placing an opaque mask at the back of the objective, you could reduce the resolution of the lines of the grating—or any other detailed object—or "destroy" the resolution altogether so that the specimen would not be visible. Hence the usual caution not to close down the condenser aperture diaphragm below the suggested ⅔–⅘ of the objective's aperture.

*Fig. 17. Diffracted Orders and Resolution*

Fig. 17



A. NO RESOLUTION

B. RESOLVED

C. BETTER RESOLUTION
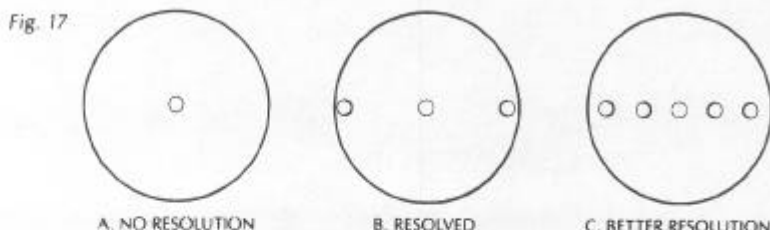
Failure of the objective to "grasp" any of the diffracted orders results in an unresolved image. (Fig. 17) Since, in a specimen with very minute details, the diffraction fans are spread at a very large angle, a high numerical aperture objective is needed to "capture" them. Likewise, since the diffraction fans are compressed in immersion oil or in water, objectives designed for such use can give better resolution than dry objectives.

If alternate diffracted orders are blocked out (still assuming the grating as our specimen), the number of lines in the grating would appear doubled—a spurious resolution. (Fig. 18) The important caveat is that actions introduced at the back of the objective can have significant effect upon the eventual image produced.

Fig. 18. 1st Order
Blocked Out.
Spurious Doubling
of Lines of Grating
at Intermediate
Image Plane of
Eyepiece.

Fig. 18



EYEPIECE
INTERMEDIATE
IMAGE
PLANE

OBJECTIVE
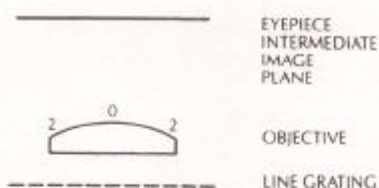
LINE GRATING

## AIRY DISKS AND IMAGE FORMATION

For small details in a specimen (rather than a line grating), the objective projects the direct and diffracted light onto the IMAGE plane of the eyepiece diaphragm in the form of small, circular diffraction disks known as Airy disks. High numerical aperture objectives "capture" more of the diffracted orders and produce smaller size disks than do low aperture objectives. (Fig. 19) The resulting image at the eyepiece diaphragm level is actually a mosaic of Airy disks which you perceive as light and dark. Where

Fig. 19.
Diffraction patterns
for circular
apertures of
decreasing size.

Fig. 19



two disks are too close together so that their central black spots overlap considerably, the two details represented by these overlapping disks are not resolved or separated and thus appear as one. (Fig. 20)

Fig. 20A & B.
Airy Disks and
Resolution

Fig. 20A

Fig. 20B



TWO AIRY DISKS
SEPARATED

TWO AIRY DISKS
NOT SEPARATED
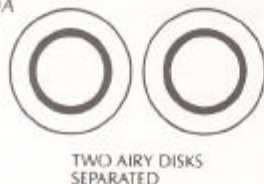
## KEY PRINCIPLE IN IMAGE FORMATION

The basic principle is that the combination of direct and diffracted light—or the MANIPULATION of direct or diffracted light—is critically important in image formation. The key places for such manipulation are the back focal plane of the objective and the front focal plane of the substage condenser. This principle is fundamental to most of the contrast improvement methods described in this booklet; it is of particular importance at high magnification of small details close in size to the wave length of light.* Abbe was a pioneer in developing these concepts to explain image formation of absorbing or so-called amplitude specimens. In the 1930's, F. Zernike, a Dutch physicist, extended these principles when he devised and explained phase microscopy.

*For specimens with openings or details much larger than the wave length of light, the angle of the diffracted rays is small. Such rays are readily captured by objectives of modest numerical aperture.

# Contrast Methods

Top left: Cartilage,
Embryonic Mouse,
unstained
40X Olympus S
Plan Objective
Magnification
132X
Differential
Interference
Contrast
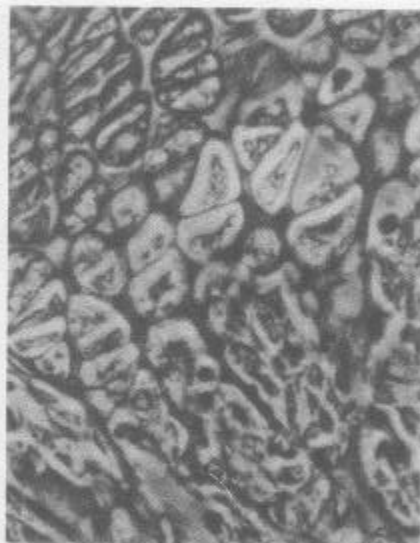
Top right: Same
View
40X Olympus S
Plan Phase
Objective
Positive (dark)
Phase Contrast

Bottom left: Dog
Tongue, unstained
40X Olympus D
Plan Objective
Magnification
132X
Hoffman
Modulation
Contrast
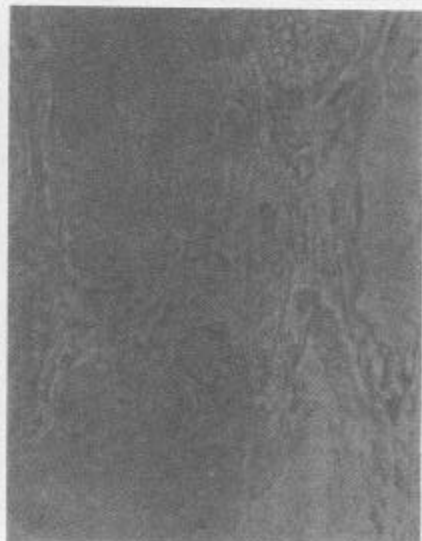
Bottom right: Same
View
40X Olympus D
Plan Objective
Brightfield
Illumination

## COLORED CHEMICAL STAINING AND COLOR FILTERS

An early and currently used method of increasing contrast of *stained* specimens employs color contrast filters placed in the light path, gelatin squares (Kodak) or glass or interference-type filters (many independent as well as microscope manufacturers). For example, if a specimen is stained with a red stain, a green filter will darken the red areas thus increasing the contrast. On the other hand, a green filter would lighten any green stained area. Microscopists can secure a set of color filters which are valuable for observation and for black-white photomicrography (see Kodak booklet "Photography Through the Microscope," pages 60-64). Green filters are particularly valuable for use with achromat and phase contrast objectives. Achromats are spherically corrected for green light; phase contrast objectives are usually designed for manipulation of wave length assuming the use of green light since phase specimens usually are transparent and without inherent color.

## REFRACTIVE INDEX OF MOUNTING MEDIA

Another simple technique for contrast improvement, this for *unstained* specimens (e.g. crystals, diatoms), is to mount the specimen in a medium with a refractive index different from that of the specimen itself. For example, diatom enthusiasts use a mounting medium such as air, Styrax or Realgar. The difference in refractive indices improves the contrast of these colorless objects and renders their outlines and markings more visible.

## DARKFIELD MICROSCOPY

All of us are quite familiar with the appearance and visibility of stars on a dark night, this despite their enormous distances from earth. The stars can be seen because of the stark contrast between the faint light and the black sky. Yet stars are shining both night and day; they are invisible during the day because the overwhelming brightness of the sun "blots out" the faint light from the stars rendering them invisible. During a total solar eclipse, the moon moves between the earth and the sun blocking out the light of the sun—the stars now can be seen even though it is daytime; the visibility of the faint star light is enormously enhanced against a dark background.

This principle is applied in darkfield (also called darkground) microscopy, a simple and popular method for making *unstained* objects clearly visible. Such objects are often barely seen in conventional brightfield microscopy.

Darkfield illumination requires the blocking out of the central light which ordinarily passes through or around (the surround) the specimen—and allowing only oblique rays to "strike" the specimen mounted on the microscope slide. (Fig. 21)

If no specimen is present AND the numerical aperture of the condenser is greater than that of the objective, the oblique rays from the condenser will cross and all such rays will miss entering the objective because of their obliquity. The field of view will appear dark.

When a specimen is placed on the slide, especially an *unstained*, non-light absorbing specimen, the oblique rays cross the specimen and are diffracted or refracted so that these faint rays are able to enter the objective; the specimen can then be seen bright on an otherwise black background. As in the example of starlight described above, the visibility is greatly enhanced by the contrast between the brightly shining specimen and the dark surround.

What has happened in darkfield illumination is that all the ordinarily undeviated rays of the zeroth order have been blocked; the oblique rays, now diffracted by the specimen and yielding 1st, 2nd, and higher diffracted orders at the back focal plane of

Fig. 21A

Fig. 21B

SPECIMEN

SPECIMEN

BRIGHTFIELD

CENTRAL RAYS BLOCKED
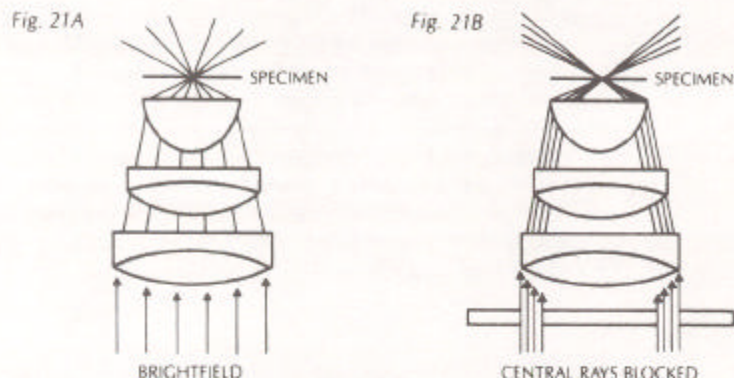
the objective, proceed onto the image plane where they interfere with one another to produce an image of the specimen. (Fig. 22)
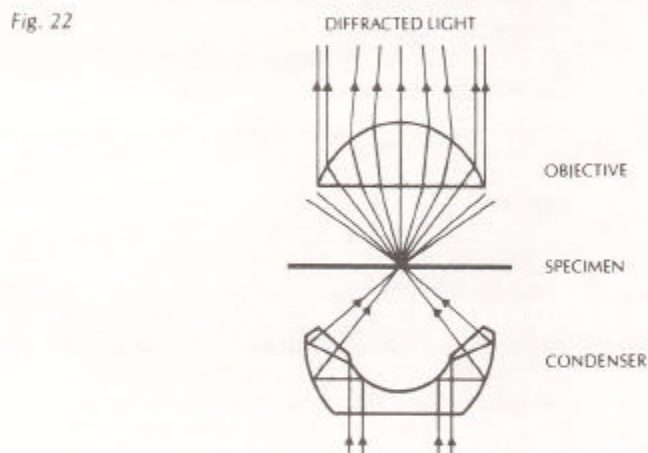
Fig. 22

DIFFRACTED LIGHT

OBJECTIVE

SPECIMEN

CONDENSER

If you were to look at the back of the objective, it would appear filled with light. This diffracted faint light is reconstituted into the visible image at the plane of the eyepiece diaphragm with its contrast reversed, bright image on black background. Since darkfield microscopy eliminates the bright undeviated light, this form of illumination is very wasteful of light and thus demands a high intensity illumination source. Microscrope slides must be of the appropriate thickness, approximately one millimeter $\pm$ 0.1; and the slides must be scrupulously clean because every dirt speck will be mercilessly bright.

There are several pieces of equipment that are utilized to produce darkfield illumination. The simplest is a "spider stop" placed just under the bottom lens (front focal plane) of the substage condenser; the aperture diaphragm is opened wide to pass oblique rays. (Fig. 23 A, B) The central opaque stop (you can make one by mounting a coin on a clear glass disk) blocks out the central rays. This device works fairly well, even with the Abbe condenser, with the 10X objective up to the 40X with an N.A. of 0.65. The diameter of the opaque stop should be approximately 8mm for the 10X objective of N.A. 0.25 to approximately 15mm for objectives of N.A. 0.65.

Fig. 23A

OBJECTIVE

SPECIMEN

SPIDER STOP

Fig. 23B

SPIDER STOP—TOP VIEW
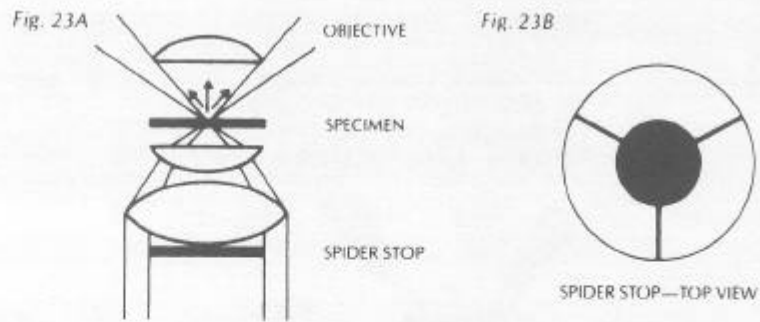
For more precise work and blacker backgrounds, you may choose a condenser designed especially for darkfield, i.e. to transmit only oblique rays. There are several varieties: dry darkfield condensers with air between the top of the condenser and the underside of the slide—and immersion darkfield condensers which require the use of a drop of immersion oil (some are designed to use water instead) establishing contact between the top of the condenser and the *underside* of the specimen slide. (Fig. 24)

Fig. 24. Oil
Immersion
Darkfield
Condenser

Fig. 24

OBJECTIVE
OBLIQUE RAYS

DIFFRACTED RAYS
SPECIMEN

OIL →

IMMERSION
DARKFIELD
CONDENSER
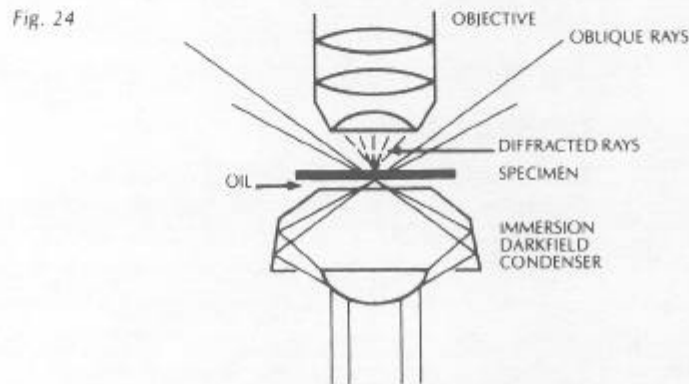
The dry darkfield condenser is useful for objectives with numerical apertures below 0.75; the immersion condenser can be used with objectives up to N.A. 1.4. The objectives with N.A. above 1.2 will require a reduction of their aperture since their N.A. may exceed the N.A. of the condenser, thus allowing direct light to enter the objective. For this reason, high N.A. objectives used for darkfield as well as brightfield are made with a built-in adjustable iris diaphragm. The immersion darkfield condenser has internal mirrored surfaces and passes rays of great obliquity free of chromatic aberration; it gives best results and blackest background.

Darkfield objects are quite spectacular to see (e.g. try a drop of fresh blood in darkfield); objects of very low contrast in brightfield shine brilliantly in darkfield. Such illumination is best for revealing outlines, edges, and boundaries; darkfield illumination is less useful in revealing internal details.

## RHEINBERG ILLUMINATION

A striking variation of low to medium power darkfield is known as Rheinberg illumination, first demonstrated by the British microscopist Julius Rheinberg nearly a hundred years ago. This method produces beautiful colored images of unstained objects. (cover photograph) In this form of illumination, the central opaque stop is replaced with a

transparent, colored, circular stop inserted into a transparent ring of a contrasting color. (Fig. 25A,B) These stops are placed under the bottom lens of the condenser; the specimen is rendered in the color of the ring; the background is the color of the central spot. For example, a green central stop inside a red ring will show "red protozoa" swimming in a "green sea." (See Needham's "Practical Use of the Microscope" pages 281-285 for further information.)
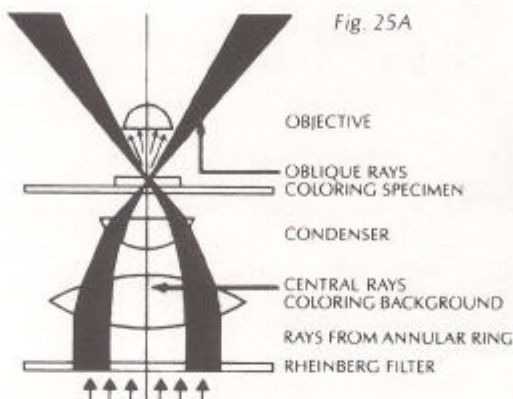
Fig. 25A&B.
Rheinberg
illumination.

Fig. 25A

OBJECTIVE

OBLIQUE RAYS
COLORING SPECIMEN

CONDENSER

CENTRAL RAYS
COLORING BACKGROUND

RAYS FROM ANNULAR RING

RHEINBERG FILTER

Fig. 25B

ANNULAR RING.
E.G. RED

CENTRAL STOP.
E.G. GREEN
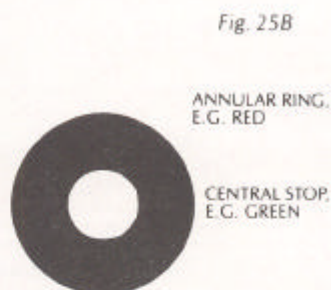
## OPTICAL STAINING

Darkfield illumination and Rheinberg illumination are examples of how images are affected by manipulating light at the substage condenser. When an image is made to appear in color without use of chemical stains, the technique is described as "optical staining."

## OBLIQUE ILLUMINATION

If you have ever examined a coin in direct bright light, you must have observed that the relief of the coin was very difficult to see. If, on the other hand, light is arranged to "strike" the coin at a low glancing angle, the resulting shadow effect on one side and brightness on the side nearer the light cause the relief detail of the coin to stand out in 3-dimensional clarity.

A somewhat similar appearance is produced with microscopic specimens with a technique known as oblique illumination. Direct light from one azimuth of the condenser light cone is allowed to illuminate the specimen, from one side only. (Fig. 26) The net effect is to reveal details in an otherwise almost invisible, colorless specimen in pseudo-relief.

Fig. 26. Oblique
Light

Fig. 26

CONDENSER
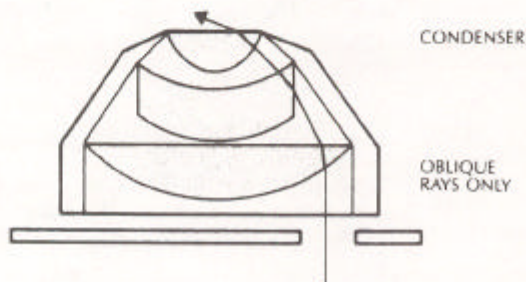
OBLIQUE
RAYS ONLY

The oblique lighting has caused the zeroth order to be moved to a position just within the periphery of the objective (this can be observed with a focusing telescope looking at the back focal plane of the objective). The shifting of the zeroth order to the periphery allows additional diffracted orders (or sometimes just one diffracted order) to be included in the objective's back focal plane. (Fig. 27) These diffracted orders are on only one side of the zeroth order; because of the obliquity, the diffracted orders on the other side "miss" the objective altogether.
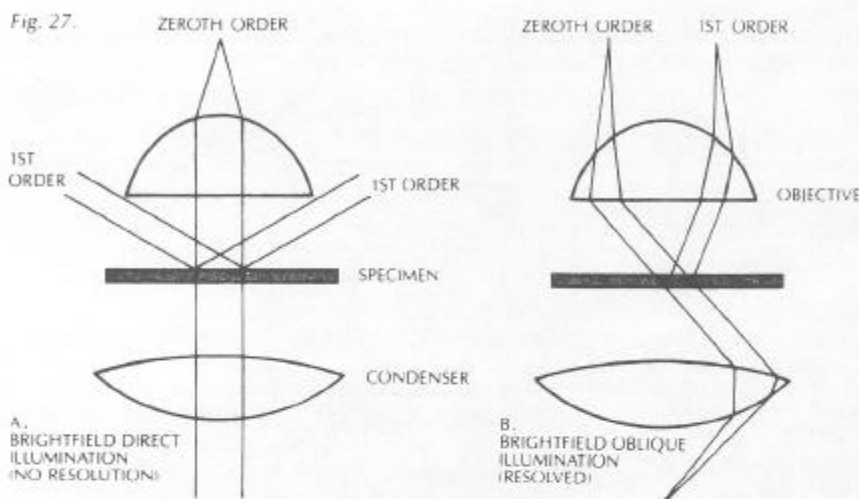
*Fig. 27. Oblique Illumination and Resolution*



Fig. 27.

ZEROTH ORDER

1ST ORDER

1ST ORDER

SPECIMEN

CONDENSER

A.
BRIGHTFIELD DIRECT
ILLUMINATION
(NO RESOLUTION)

ZEROTH ORDER    1ST ORDER

OBJECTIVE

B.
BRIGHTFIELD OBLIQUE
ILLUMINATION
(RESOLVED)

The result may be to increase the resolution and also to produce a shadowed, relief-like pseudo 3-dimensional appearance in the image of the specimen. This method works well with many unstained objects, e.g., living cells, crystals, diatoms, etc. The resulting image must be viewed with caution because the diffracted orders from one side have not contributed to the image formation.

To achieve oblique illumination, you place a so-called sector stop just below the lower lens of the condenser (a filter holder will do fine for holding the stop). (Fig. 28A) The cut out section of the sector stop allows only oblique, direct light to pass through to the specimen.

*Fig. 28A. Sector Stop*



Fig. 28A.

APPEARANCE AT
BACK OF OBJECTIVE

In former years, some microscopes were equipped with a condenser with decentera-ble aperture iris diaphragm. The device was engineered to allow the entire iris to move off center in a horizontal plane so that closing the iris down would result in moving the zeroth order to the periphery of the back focal plane of the objective—a similar effect as the sector stop described above. The entire diaphragm was rotatable around the axis of the microscope so that oblique light could be directed toward the specimen from any azimuth to achieve the best desired effect for a given specimen. (Fig. 28B)

Here again, you find that the manipulation of light at the level of the aperture iris diaphragm (front focal plane of the condenser) can significantly alter the appearance of the IMAGE at the position of the eyepiece fixed diaphragm.

Fig. 28B.
Condenser with
Decenterable Iris
Diaphragm

CONDENSER

APERTURE
DIAPHRAGM
LEVER

HORIZONTAL DECENTERING KNOB

MIRROR

## PHASE CONTRAST MICROSCOPY

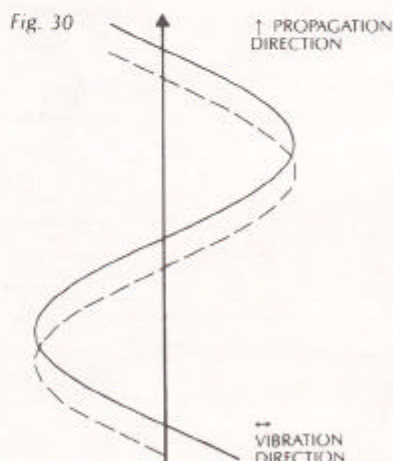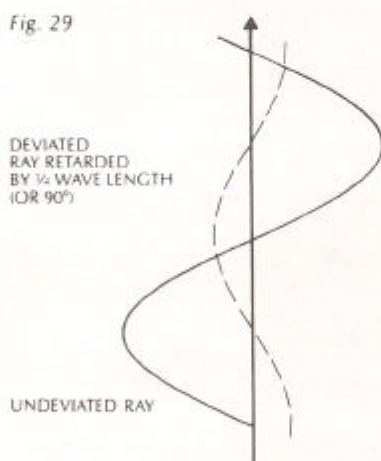The search was still on in the 1930's to find a way of using both direct and diffracted light from all azimuths to yield good contrast images of unstained objects that do not absorb light. Such specimens are called phase objects because they slightly alter the phase of the light diffracted by the specimen, usually by retarding such light approximately a 1/4 wave length as compared to the undeviated direct light passing through or around the specimen unaffected. (Fig. 29) Unfortunately, our eyes as well as camera film are unable to detect these phase differences. To reiterate, our eyes are sensitive to differences in intensity (related to wave amplitude) or to color (frequency).

Fig. 29. Phase
Object—
Brightfield
Microscopy wave
action—Schematic
Prior to Arrival at
Image Plane. Solid
line represents
undeviated ray.
Dotted line
represents deviated
ray caused by
particle having
slightly different
refractive index

Fig. 30. Phase
Object—
Brightfield
Microscopy Wave
Action—
Schematic Arrival
at Image Plane—
Localized Area
Solid line
represents
undeviated rays
and background.
Dotted line
represents resultant
of undeviated ray
and ray deviated
1/4 λ by phase
specimen. Note
amplitude almost
identical. Hence
little or no contrast.

Fig. 29

DEVIATED
RAY RETARDED
BY ¼ WAVE LENGTH
(OR 90°)

UNDEVIATED RAY

Fig. 30

↑ PROPAGATION
DIRECTION

↔ VIBRATION
DIRECTION

In phase specimens, the direct zeroth order light passes through or around the specimen undeviated. However, the light diffracted by the specimen is not reduced in amplitude as it is in a so-called amplitude object; (see previous sections) but is slowed by the specimen because of the specimen's refractive index or thickness (or both). This diffracted light, lagging behind by approximately 1/4 wave length, arrives at the image plane out of step with the undeviated light but essentially undiminished in intensity. (Fig. 30) The result is that the image at the eyepiece level is so lacking in contrast as to make the details almost invisible.

F. Zernike succeeded in devising a method—phase contrast microscopy—for making unstained, phase objects yield contrast images AS IF they were amplitude objects. Since amplitude objects show excellent contrast when the diffracted and direct light are "out of step" (phase difference) by 1/2 wave length, Zernike's method was to "speed up" the direct light by 1/4 wave length so that the difference in wave length between the direct and diffracted light for a PHASE SPECIMEN would now be 1/2 wave length. (Fig. 31) As a result, the direct and diffracted light arriving at the image level of the eyepiece would be able to produce destructive interference (see section on image formation for absorbing objects previously described). Such a procedure results in the details of the image appearing darker against a lighter background; it is called dark or positive phase contrast.

Fig. 31A
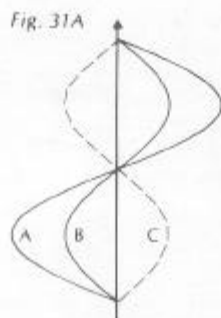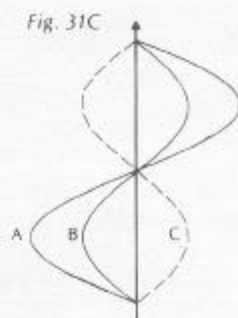
A = UNDEVIATED LIGHT
B = RESULTANT OF
    DESTRUCTIVE
    INTERFERENCE
C = DIFFRACTED LIGHT
    OUT OF PHASE 1/2 λ
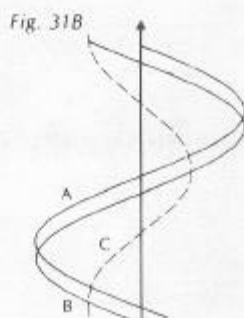
Fig. 31B

A = UNDEVIATED LIGHT
B = RESULTANT OF
    UNDEVIATED LIGHT (A)
    AND DIFFRACTED
    LIGHT (C)
C = DIFFRACTED LIGHT,
    OUT OF PHASE 1/4 λ
    OR 90°

Fig. 31C

A = UNDEVIATED LIGHT
    "SPEEDED UP" 1/4 λ
    BY PHASE SHIFTER
B = RESULTANT OF SHIFTED
    UNDEVIATED LIGHT
    AND DIFFRACTED LIGHT
    —DESTRUCTIVE
    INTERFERANCE
C = DIFFRACTED LIGHT
    RETARDED BY 1/4 λ

Another possible course, much less often used, is to arrange to "slow up" the DIRECT LIGHT by 1/4 wave length so that the diffracted light and the direct light arrive at the eyepiece in step and can interfere constructively. This arrangement results in a bright image of the details of the specimen on a darker background; it is called negative or bright contrast.

Phase contrast microscopy was most successful, and, as a result, Zernike was eventually awarded the Nobel prize in physics in 1953. This method was hailed as the greatest advance in microscopy in a century. Phase contrast, by "converting" phase specimens such as living material into amplitude specimens allowed scientists to see details in unstained and/or living objects with a clarity and resolution never before achieved.

The Zernike method involves the "separation" of the direct zeroth order light from the diffracted light at the back of the objective. To do this, a ring annulus is placed in position directly under the lower lens of the condenser (front focal plane of the condenser). As the hollow cone of light from such an annulus passes through the specimen undeviated, it arrives at the back focal plane of the objective in the shape of a ring of light. The fainter light diffracted by the specimen is spread all over the back focal plane of the objective.

If this combination were allowed, as is, to proceed to the image plane of the eyepiece, the diffracted light would be approximately 1/4 wave length behind the direct light; at the image plane, the phase of the diffracted light would be out of step with the direct light but its amplitude would be almost the same as that of the direct light; the result would be little contrast.

To "speed up" the direct undeviated zeroth order light, Zernike installed a phase plate with a ring shaped "phase shifter" on it at the back focal plane of the objective; the narrow ring-shaped area of the phase plate is THINNER than the rest of the plate. (Fig. 32) As a result the undeviated light passing through the phase ring travels a shorter distance in traversing the objective than does the diffracted light.

Fig. 32. Longi-
section—Phase
Microscope Dark
Contrast



Fig. 32

REAR FOCAL PLANE—PHASE PLATE

OBJECTIVE

SPECIMEN

FRONT FOCAL PLANE OF CONDENSER—ANNULUS

FULL VIEW OF PHASE PLATE

FULL VIEW OF ANNULUS
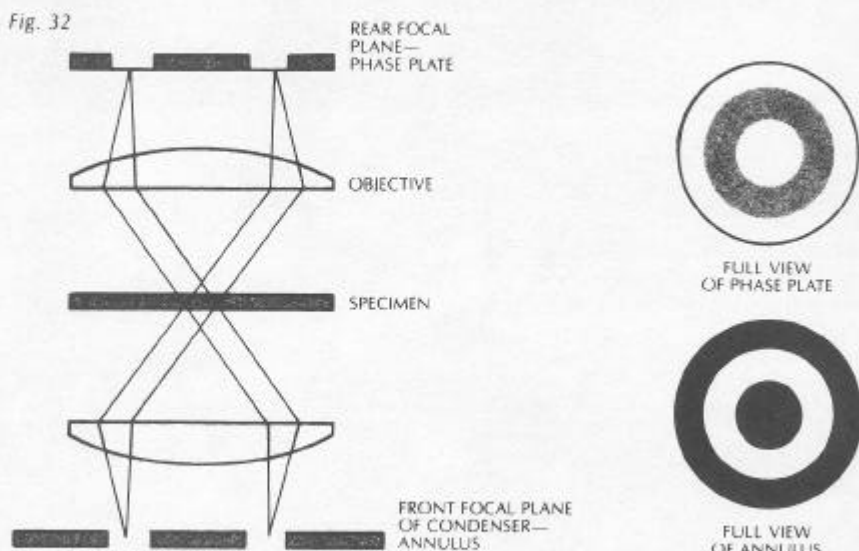
Now, when the direct undeviated light and the diffracted light proceed to the image plane, they are 1/2 wave length out of phase with each other. The diffracted and direct light can now interfere DESTRUCTIVELY so that the details of the specimen appear dark against a lighter background (just as they do for an absorbing or amplitude specimen). (Fig. 33) This is a description of what takes place in *positive or dark phase contrast*.

Fig. 33. Destructive
Interference—
Dark Contrast
(Positive)
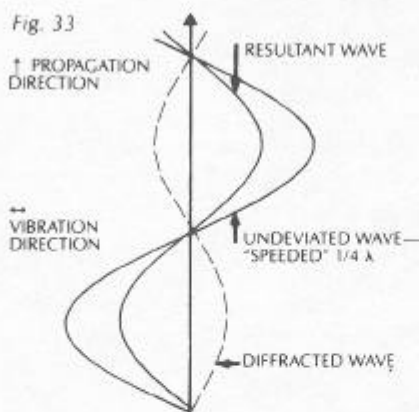
Fig. 34. Constructive
Interference—
Bright Contrast
(Negative)



Fig. 33

↑ PROPAGATION DIRECTION

↔ VIBRATION DIRECTION

RESULTANT WAVE

UNDEVIATED WAVE—"SPEEDED" 1/4 λ

DIFFRACTED WAVE

Fig. 34

RESULTANT WAVE

UNDEVIATED WAVE—SLOWED 1/4 λ
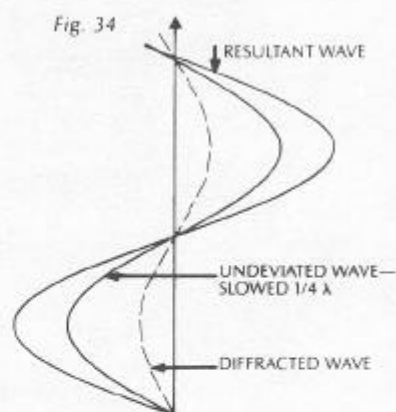
DIFFRACTED WAVE

If the ring "phase shifter" area of the phase plate were to be made thicker than the rest of the plate, direct light would be slowed by 1/4 wave length; it would arrive at the image plane in step with the diffracted light; CONSTRUCTIVE interference would take place. (Fig. 34) The image would appear bright on a darker background. This describes *negative or bright contrast*.

Since the undeviated light of the zeroth order is much brighter than the faint diffracted light, a thin absorptive transparent metallic layer is deposited on the ring to bring the direct and diffracted light into better balance of intensity to increase contrast. Also, since the "speeding up" or "slowing down" of the direct light is calculated on a 1/4 wave length of green light, the phase image will appear best when a green filter is placed in the light path; a green interference filter is preferable. Such a green filter also helps achromatic objectives produce their best images since achromats are spherically corrected for green light.

The accessories needed for phase contrast work are a substage phase contrast condenser equipped with annuli and a set of phase contrast objectives, each of which has a phase plate installed. The condenser usually has a brightfield position with aperture diaphragm and a rotating turret of annuli (each phase objective of different magnification requires an annulus of increasing diameter as the magnification of the objective increases). Each phase objective has a darkened ring on its back lens. Such objectives can also be used for ordinary brightfield transmitted light work with only slight reduction in image quality.

The phase outfit usually also includes a green filter and a phase telescope. (Fig. 35) The latter is used to enable you to align the condenser annulus to "superimpose" it on the ring of the phase plate. You will manipulate the annulus centering screws to align the annulus while you observe the back focal plane of the objective with the telescope.

Fig. 35. Phase Outfit

Fig. 35



GREEN FILTER
PHASE TELESCOPE
PHASE CONDENSER
PHASE OBJECTIVES
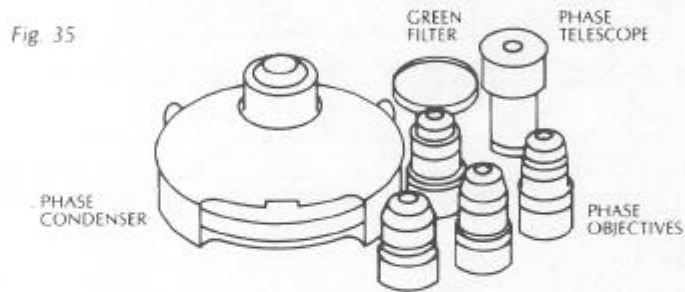
To set up phase microscopy (cheek lining cells are a readily available test material), focus the specimen with the 10X phase objective. Set up Koehler illumination using the brightfield (0) position of the condenser; this is to assure the proper alignment of the microscope's objective, condenser, and field diaphragm. Then open up the condenser aperture diaphragm and swing the turret of the condenser into the 10 position (this usually automatically opens the aperture diaphragm). Place the green filter in the light path, and remove one of the eyepieces. Insert the phase telescope* and, while observing the back of the objective, use the ANNULUS CENTERING SCREWS to center the annulus to the ring of the phase plate. (Fig. 36) This centering is often easier to do if the specimen is out of the light path temporarily. Then put back the regular eyepiece.

Fig. 36. Phase Plate and Light Annulus.

Fig. 36



PRIOR TO ALIGNMENT

AFTER ALIGNMENT
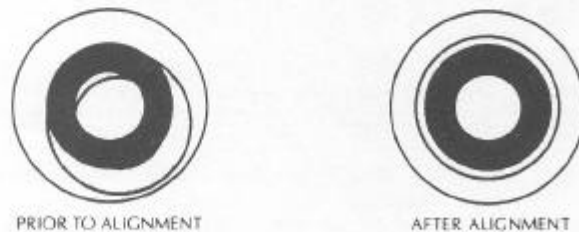
*A Bertrand lens or a pinhole eyepiece, if available, can serve for viewing the back focal plane of the objective.

This same procedure is followed for each objective making sure that you have rotated the turret to match the objective magnification.

Some manufacturers, e.g. Olympus, provide individual push-in, centering, annuli that can be inserted into the lower part of the common Abbe condenser. Such inexpensive, simple devices do well with the 10X, 20X, and 40X phase objectives but the condenser can receive only one at a time.

Phase microscopy continues to be a most important tool, particularly for the microscopist studying living and/or unstained material. This method is also currently used simultaneously with reflected light fluorescence to reveal areas of a specimen that do not fluoresce. Phase microscopy techniques are particularly useful with specimens that are thin and scattered in the field of view.

You should also be aware of some of the limitations of phase microscopy:

Phase images are usually surrounded by halos around the outlines of details. Such halos are optical artifacts which sometimes obscure the boundaries of details.

The annuli do limit the working numerical aperture somewhat, thus reducing resolution.

Phase contrast does not work well with thick specimens because of shifts in phase from areas just below or just above the plane you may be focusing on.

Phase images appear gray if white light is used; green if a green filter is used. Thus, in phase, you are restricted to black and white film for photomicrography; you cannot get the "eye impact" of a color image.

Phase microscopy is another exemplification of how the manipulation of light at the substage condenser lower lens level and at the objective rear focal plane level has significant effect upon the image that you observe through the eyepiece.


## DIFFERENTIAL INTERFERENCE CONTRAST MICROSCOPY

In the past twenty-five years, another method of microscopy—differential interference contrast or DIC—has become very widely used. A particular version, in accordance with the design suggested by George Nomarski (a French optics theoretician), will be described here. In DIC too, living or unstained material, yielding images of poor contrast in ordinary brightfield transmitted light, is made visible by optical rather than chemical means. The advantages as well as limitations of DIC will be dealt with later.

In transmitted light DIC, light from the light source is passed through a polarizer* located below the substage condenser. (Fig. 37) Next in the light path of this plane

*Fig. 37 Random Light Rays Incident on a Polarizer*

Fig. 37



LIGHT RAYS WITH RANDOM ORIENTATION OF VIBRATION PLANE

LIGHT RAYS TRANSMITTED BY POLARIZER HAVE THE SAME PLANE OF VIBRATION
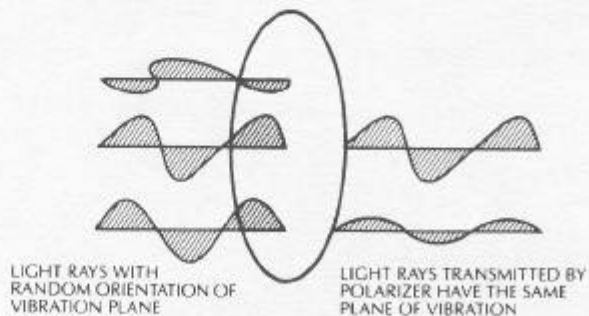
polarized light is a modified Wollaston** prism located below the lower lens of the condenser. A different prism is needed for each objective of different magnification. A revolving turret on the condenser allows you to rotate the appropriate prism into the light path.

The plane polarized light, VIBRATING only in one direction perpendicular to the propagation direction of the light beam, enters the BEAM-SPLITTING modified Wollaston prism and is split into two rays, vibrating perpendicular to each other. These two rays travel close together but in slightly different directions. (Fig. 38) The rays
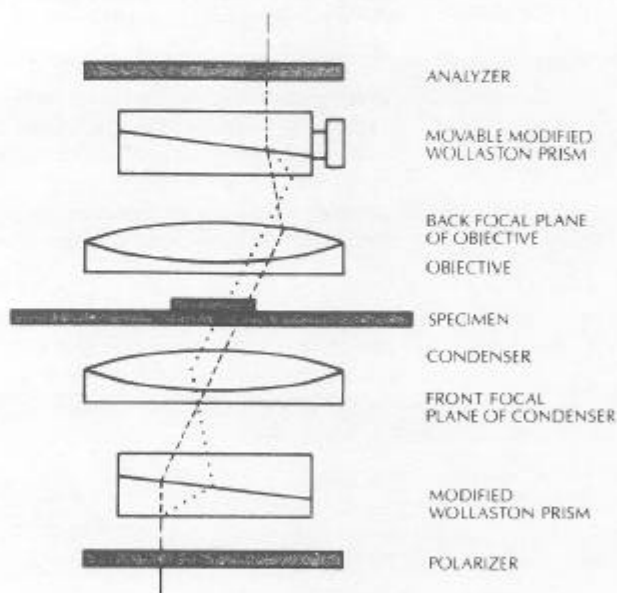
*Fig. 38 Modified Wollaston Prism*

Fig. 38



FRONT FOCAL PLANE OF CONDENSER
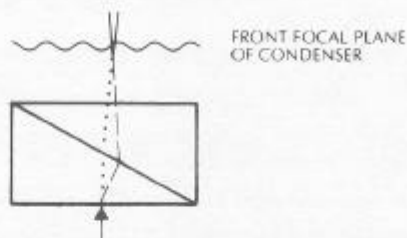
intersect at the front focal plane of the condenser. The two rays pass out of the condenser, traveling PARALLEL and extremely close together, and with a slight path difference. The distance between the rays, called the shear, is so minute that it is less than the resolving ability of the objective. The rays emerge from the condenser still VIBRATING perpendicular to each other and therefore unable to interfere with one another. (Fig. 39)

*Fig. 39 Schematic Longi-Section Differential Interference Microscope*

Fig. 39



ANALYZER

MOVABLE MODIFIED WOLLASTON PRISM

BACK FOCAL PLANE OF OBJECTIVE

OBJECTIVE

SPECIMEN

CONDENSER

FRONT FOCAL PLANE OF CONDENSER

MODIFIED WOLLASTON PRISM

POLARIZER

*The polarizer is usually a plastic sheet of high grade Polaroid-type material which allows light *vibrating* in only *one* plane perpendicular to the light path to pass through. An analyzer is similarly constructed.

**The modified Wollaston prism "splits" an entering beam of polarized light into two beams traveling in slightly different directions. The prism is made of two halves cemented together. The emerging rays *vibrate* at 90 degrees to each other with a slight path difference.

The "split" beams enter and pass through the specimen. The specimen alters the wave paths in accordance with the specimen's varying thicknesses, slopes and refractive indices. These variations cause alterations in the wave path of both beams passing through areas of the specimen details lying close together. (Fig. 40) The parallel beams are still proceeding up through the microscope into the objective where they are focused at the back focal plane of the objective. Here the beams enter the upper BEAM COMBINING modified Wollaston prism which removes the shear between the beam pairs and the original path difference. As a result of having traversed the specimen, the *paths* of the parallel beams are not of the same length (optical path difference) for differing areas of the specimen.

Fig. 40 Specimen
Altering Wave
Paths of Adjacent
Areas According to
Thickness and
Refractive Index

Fig. 40
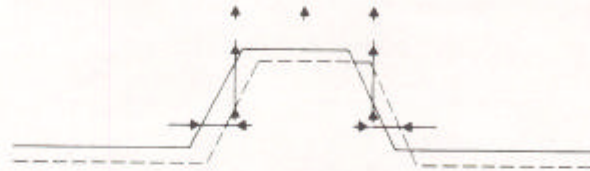


In order for the beams to interfere, the vibrations of the beams of different path length must be brought into the same plane and axis (NOT as before vibrating perpendicular to each other). This is accomplished by placing an analyzer above the upper beam combining prism. The light then proceeds toward the eyepiece. In white light, the optical path differences are then seen as differences in intensity and differences in color; the *optical color staining* is quite striking.

The design results in one side of a detail appearing bright (or possibly in color) while the other side appears darker (or another color). This shadow effect is *pseudo* 3-dimensional.

In some instruments, the upper modified Wollaston prism is combined in one fitting with the analyzer incorporated above it. The upper prism may also be arranged so that it can be moved horizontally. This allows for varying optical path difference by moving the prism; *alteration* of brightness and color of the background and specimen is thus controlled by the user. Because of the design of the prisms and their placement, the *background* will be homogeneous for whatever color has been selected.

To sum up the equipment needed: a polarizer, a beam-splitting modified Wollaston prism below the condenser, a beam-recombining modified Wollaston prism above the objective, and an analyzer above this upper prism. Individual prisms are required (for each objective) below the condenser. For the upper prism, a single prism serves for all objectives. The upper prism can be moved laterally. (Fig. 40A)

Fig. 40A
Differential
Interference
Contrast Outfit

Fig. 40A



DIC CONDENSER

INTERMEDIATE PIECE

FOCUSING TELESCOPE

BRIGHTFIELD OBJECTIVES
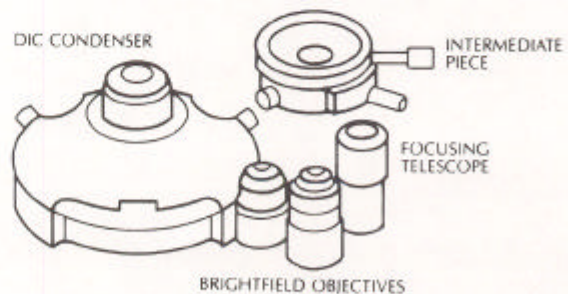
The distance of the placement of the lower prism in relation to the front focal plane of the condenser and the distance of the upper prism from the back focal plane of the objective are quite critical. Manufacturers therefore designate which of their objectives are suitable for their particular DIC apparatus.

The color and/or light intensity effects shown in the image are related especially to the *rate of change* in REFRACTIVE INDEX, THICKNESS OR BOTH in details or adjacent areas of the specimen. The image appears 3-dimensional. This appearance is NOT representing the true geometric nature of the specimen but is an exaggeration based on "optical thickness."* It is not suitable for accurate measurement of actual heights and depths.

At the gray setting of the movable upper prism, the 3-dimensionality is most marked. The orientation of the specimen can significantly improve the relief-like appearance. Sometimes the rotation of the specimen 180 degrees changes a "hill" into a "valley" or vice versa; hence the interpretation of the image must be done with caution. The darker appearance on one side and the lighter appearance on the other side of a detail greatly improve the visibility by giving a pseudo-relief effect.

To set up your microscope for DIC, you should take the following steps:

Fit the DIC condenser into the substage of the microscope and fit the DIC intermediate piece between the nosepiece and the viewing tubes. Using a 10X objective and the condenser at the brightfield (0) position** and polarizer in the light path, set up Koehler illumination with your specimen in place. Move the specimen out of the light path and remove one of the eyepieces.

Insert a phase focusing telescope into the eyepiece tube and, while looking at the back focal plane of the objective, rotate the screw of the UPPER prism until you see a diagonal blackish line appearing at the center of the back of the objective. Now slightly rotate the substage POLARIZER to make the black line appear as black as possible. This, in effect, is adjusting the polarizer so that it is "crossed" (at a 90 degree angle) with the analyzer that is situated above the upper prism. Make sure the condenser aperture diaphragm is open to 2/3 to 4/5 of the back lens diameter of the objective.

Remove the focusing telescope and put back the regular eyepiece. Rotate the turret of the condenser to bring the appropriate lower prism into the light path; this is usually marked by the red 10 setting on the turret. Move the specimen back into the light path. Now you may use the knob of the upper prism to move it to and fro laterally to achieve the desired effect or color. You may also rotate the stage to change the orientation of the specimen to improve the effect.

Similar steps are taken for each objective being used, making sure you set up Koehler illumination for each objective in turn by adjusting both the field and aperture diaphragms.

There are numerous advantages in DIC microscopy as compared particularly to phase microscopy:

It is possible to make fuller use of the numerical aperture of the system because there is no substage annulus to restrict the aperture; Koehler illumination is properly utilized.

*"Optical thickness" refers to changes in light path resulting from change in refractive index or actual thickness or some combination of both of these variables.

**The DIC condenser usually contains four prisms, a brightfield opening with aperture diaphragm for regular brightfield work, and several light annuli. The light annuli, together with phase objectives, enable you to make quick comparisons between phase contrast and DIC images. A rotatable polarizer is fitted below the prisms.

Use of full objective aperture enables you to focus on a thin plane section of a thick specimen without confusing images from above or below the plane you are focusing on; this is called "OPTICAL SECTIONING." Larger apertures also yield better resolution in microscopy.

There are no confusing halos as may be encountered in phase images.

Images can be seen in striking color ("OPTICAL STAINING") and in 3-dimensional shadowed-like appearance. The visibility of outlines and details is greatly improved; the photography of these images is striking in color and detail.

Regular plan achromats or achromats—also suitable for ordinary brightfield work—are used if the manufacturer states that such objectives are designed for their apparatus.

There are several disadvantages or limitations in DIC:

The equipment for DIC is quite expensive because of the many prisms that are required.

Birefringent specimens such as those found in many kinds of crystals may not be suitable because of their effect upon polarized light. Similarly, specimen carriers, such as culture vessels, Petri dishes, etc., made of plastic may not be suitable.

For very thin or scattered specimens, better images may be achieved using phase contrast methods.

Apochromatic objectives are not suitable because such objectives themselves significantly affect polarized light.

Here again, in DIC, you find that manipulation of light at the front focal plane of the condenser and at the back focal plane of the objective—in this technique with the aid of a polarizer and analyzer—has significant effect upon the appearance of the image you "see" through the eyepiece.

## MODULATION CONTRAST MICROSCOPY

Another technique for increasing visibility and contrast, especially for unstained objects and living material, is Modulation Contrast, invented by Robert Hoffman.

This method employs several accessories and is adaptable to light microscopes of all major manufacturers.

A MODULATOR, or so-called amplitude filter, is inserted on the back focal plane of an achromat or planachromat (objectives of 10X-100X are suitable). The modulator has 3 zones: a small, dark zone near the periphery of the back focal plane which transmits only 1% of light; a narrow gray zone which transmits 15%; and the remaining zone, covering the back of the objective, which transmits 100% of the light. UNLIKE the phase plate in phase microscopy, the Hoffman modulator is designed NOT to alter the phase of the light passing through any of the zones. (Fig. 41)

Below the stage, a condenser with rotating turret is utilized. The turret condenser has a brightfield (0) opening with aperture diaphragm for regular brightfield microscopy. At each of the other turret openings, there is an off-center slit which is partially covered with a small rectangular polarizer. The size of the slit with polarizer is different for each objective of different magnification; hence the need for a turret arrangement.
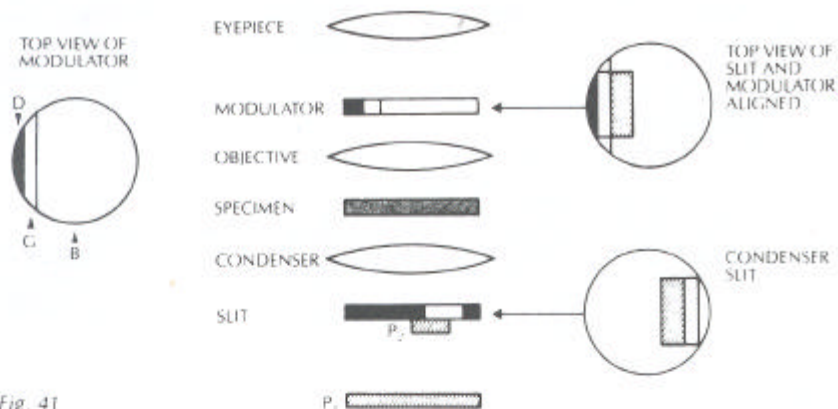
Fig. 41
Hoffman
Modulation
Contrast



TOP VIEW OF
MODULATOR

EYEPIECE

MODULATOR

TOP VIEW OF
SLIT AND
MODULATOR
ALIGNED.

OBJECTIVE

SPECIMEN

CONDENSER

CONDENSER
SLIT

SLIT

P₂

Fig. 41

P₁

The Hoffman design is such that the slits are at the front focal plane of the condenser.
When light passes through the off-axis slit, it is imaged at the back focal plane of the
objective where the modulator has been installed. (Fig. 42)

Fig. 42. Slit
Aperture
Alignment.

Fig. 42



INCORRECT

CORRECT

Below the condenser, a round polarizer is placed on the light exit port of the
microscope (note that both polarizers are BELOW the specimen). The rotation of this
polarizer can control the effective width of the slit opening; for example a "crossing" of
both polarizers at 90 degrees to each other results in "narrowing" the slit so that its
image falls within the gray area of the modulator. The part of the slit controlled by the
polarizer registers on the bright area of the modulator. As the polarizer is rotated,
contrast can be varied for best effect.

Both the modulator and the slit are offset from the optic axis of the microscope. This
arrangement permits fuller use of the numerical aperture of the objective and results in
good resolution of details. Details and shapes are rendered in shadowed, pseudo 3-
dimensional appearance; these appear brighter on one side, gray in-between, darker
on the other side, against a gray background. The modulator converts optical phase
gradients in details (steepness or slope or rate of change in refractive index or thickness
in specimen details) into changes in intensity of various areas of the image at the plane
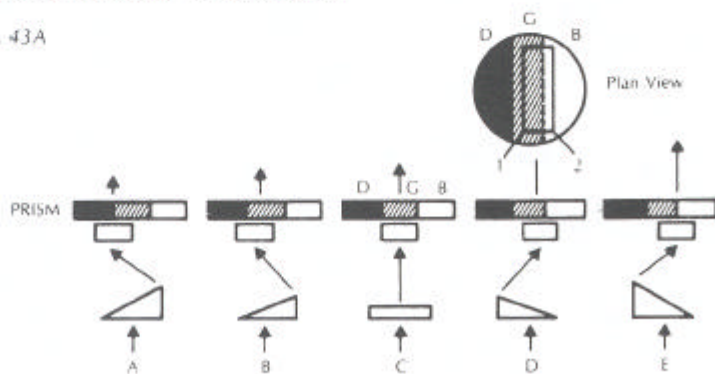of the eyepiece diaphragm. (Fig. 43)

Fig 43A. Diagram
illustrating how
the modulator
produces intensity
variations within a
range of optical
gradients. Length
of arrow after
modulator
indicates intensity.
The sum of the
intensity of area
1 and 2 is the
intensity after the
modulator. Prism
represents
idealized
specimen

Intensity Variations
produced by
Modulator.
Prism represents
an Idealized
Specimen.
Length of Arrows
above Modulator
represents
Intensity.

Fig. 43A



Plan View

PRISM

A    B    C    D    E

Opposite gradients result in deflection of the slit image to either the very dark part of the modulator or the bright section of the modulator. Any non-gradient part of the specimen and also the background (surround) register on the gray part of the modulator. The result is that the intensity of the image area from one side of a gradient is dark; the intensity from the opposite side of the gradient yields a bright image area; the non-gradient areas appear gray on the image, as does the surround.
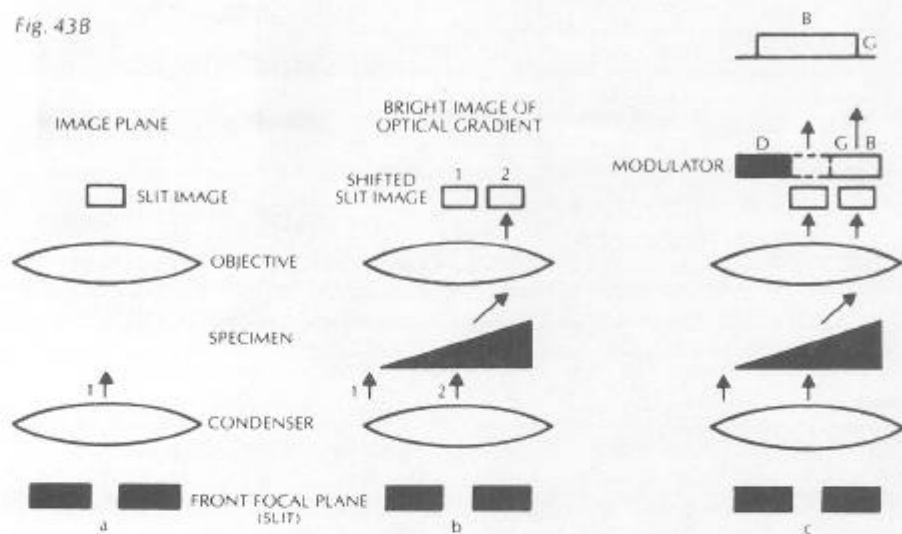
The contrast (related to variations in intensity) of the dark and bright areas against the gray gives a shadowed pseudo-relief effect. This is modulation contrast.

Rotation of the polarizer alters the contrast achieved; similarly the orientation of the specimen on the stage may improve the contrast.

Since the modulator affects the image of the slit according to how the specimen's details shift the image of the slit—and thus results in altering light intensities—it is described as an amplitude filter.

There are numerous advantages as well as limitations to Modulation Contrast.

The advantages are:

With fuller use of the numerical aperture of the objective, good resolution of details is possible; this with good contrast and visibility.

With high numerical aperture, it is possible to do "optical sectioning," that is to focus on a thin plane of the specimen without confusing images from areas above or below the plane you are focusing on.

Images appear shadowed or pseudo 3-dimensional enhancing visibility because of differences in contrast on either side of a detail. There are no halos exhibited in the image.

Achromats or planachromats can yield good images since color is not involved; a green filter under the lower polarizer will further improve the image because achromats are spherically corrected for green light.

The cost of the modulation contrast accessories is considerably below that of DIC equipment.

Birefringent objects (rock sections, crystals, bone, etc.) can be examined since the specimen is NOT between the two polarizers. Further, specimens can be contained in plastic or glass vessels without deterioration of the image because of polarization effects since such vessels are also above both polarizers, not between them.

With the condenser set at the brightfield (0) position, objectives with modulator installed can also be used for regular brightfield work; because the modulator is off-axis little deterioration of the image results.

Rotation of the polarizer enables you to vary the contrast for best effect.

These are also disadvantages and limitations:

The cost of modification of each objective and the condenser openings must be added to the basic cost of these accessories themselves. Complex, high numerical aperture, multi-element objectives are difficult or too expensive to modify. In recent months, Modulation Optics has been producing its own objectives and condensers; this reduces the range of readily available objective types. Custom work can be done.

Images must be viewed with caution because different observers can "see" a "hill" in the image as a "valley" or vice versa as the pseudo 3-dimensional image is observed through the eyepiece.

The system is most sensitive to gradients perpendicular to the length of the slit; as a result, skill is required in the orientation of the specimen for best effect.

Images are not rendered in color.

Once again, you find that manipulation of light at the front focal plane of the condenser (by means of an offset slit) and manipulation of light at the back focal plane of the objective (offset modulator) can have a significant effect upon the image you see through the eyepiece.

*By now you have "traveled through" a variety of methods for improving contrast and visibility of the microscope image. It must be clear that the image you see depends not only on the structure of the specimen but also on the way in which you manipulate the light passing through the specimen. No image is a perfect replica of the specimen because no objective can have a large enough aperture to capture all the rays of light coming from the specimen. However, microscope theorists and designers have devised a whole range of methods to produce images for you to observe. These methods used in conjunction with one another can help you to come to a more accurate understanding of the "real" structure of the specimen.*

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