

# Biomedical products

## Varel: A New Contrasting Method For Microscopy

By Becky Hohman and Ernst Keller

### Introduction

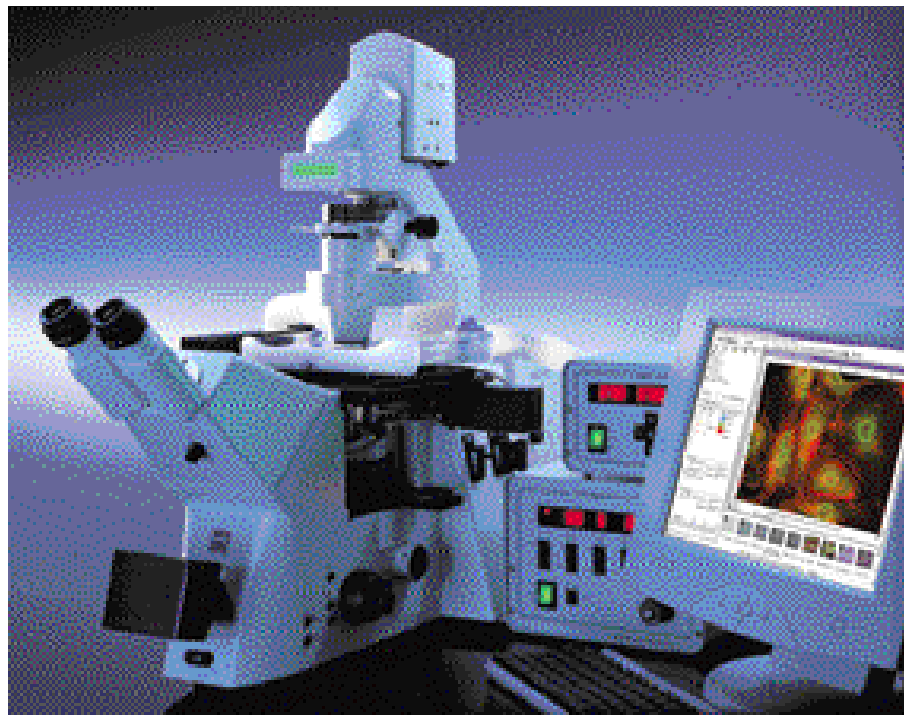
The inverted light microscope has evolved from a relatively simple instrument for tissue and cell culture into a sophisticated microscope for every discipline of science. Whether the goal is to observe growth, to manipulate either physically or biochemically, or to measure characteristics, one problem continues to plague anyone trying to view living unstained cells: how to introduce contrast while preserving resolution. Carl Zeiss has recently revolutionized the traditional tissue culture microscope with the development of a new technique, known as VAREL, which greatly enhances observation of unstained material in transmitted light. VAREL, abbreviated from *Variable Relief Contrast*, is an innovative approach to oblique illumination, a technique which dates back many years.

### Principles of image formation

To understand VAREL, one must first review Abbe's principles of image formation through the microscope, and how we manipulate that image to increase contrast. In a brightfield microscope (Figure 1), parallel light (or plane wave fronts) is sent by the condenser through the specimen and into the objective. Structures within the specimen plane cause this light to be diffracted, with large structures diffracting small angles, and small structures diffracting large angles. The ability to resolve these small structures is based upon the ability of the objective to collect their angles of dif-

fraction, which in turn is a function of the objective's collecting aperture, as measured by its Numerical Aperture. However, resolution is only part of the story. Contrast is of equal importance in the image forming system and is carefully balanced to give us as much information from the specimen as possible. If the specimen has been stained, or has inherently high absorption differences, the small and large structures not only diffract the light, but absorb the light somewhat as it passes through the speci-

men. Detail and contrast in the image are generated by interference of the diffracted wave fronts with the non-diffracted, direct source waves. Relative amplitude (intensity) and phase (wave position) differences determine the contrast. When the "optical path difference" between the undiffracted light (light directly from the light source) and the various angles of diffracted light is at least  $1/2\lambda$  and their amplitudes are of similar strength, good contrast is generated by optimum interference conditions.



A Carl Zeiss inverted light microscope.

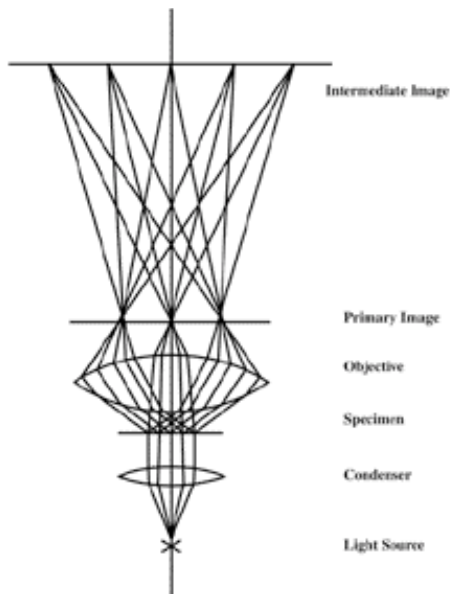


FIGURE #1 Brightfield:  
Result of Specimen Diffraction

Brightfield's use of the full numerical aperture of both the condenser and objective allows the highest resolution possible in the microscope, but particularly on unstained live material of poor contrast. Only closing the condenser diaphragm attenuates the source waves' amplitude for enhanced interference between source and diffracted waves. Although brightfield can sometimes be used for cell suspensions, it is not usable for adherent cell cultures (so-called phase specimens) because of insufficient contrast.

In 1934, Zernicke, a Dutch physicist used this basic principle as a mechanism to manipulate contrast in the light microscope. This "Phase Contrast" concept, shown schematically in Figure 2, is to separate and manipulate the non-diffracted wave fronts from the diffracted light, to enhance the interference conditions between both, yielding far better contrast. An annular ring (phase ring) is placed in the condenser's front focal plane, and a corresponding size ring (phase plate) is placed in the back focal plane of the objective. The ring located in the objective has a dense coating which allows only 10-20% light transmission. It is also slightly thinner than the rest of the phase plate and advances the non-diffracted light coming from the annular ring of the phase contrast condenser by  $1/4\lambda$ . The diffracted angles of light, conversely, freely pass outside the phase ring. By separating and shifting the source illumination, we can now "create" the  $1/2\lambda$  optical path difference, and the intensity relationship needed to achieve

good contrast for visual detection. The result is structures of higher optical path length (the combination of thickness and refractive index) appear dark and those of lower optical path length appear bright. One byproduct of phase contrast is the troublesome halos often seen in areas of drastic refractive index changes (a cell edge, for example). This is easily explained, now that one understands the image forming path. The structure itself acts as a lens and refracts some of the source light traveling adjacent to the edge, causing it to pass into the objective inside or outside the phase ring.

### Hoffman modulation contrast

The advantage of phase contrast is quite obvious. With minimal additional cost, a microscope can be equipped for phase contrast in addition to traditional brightfield. Although the illuminating numerical aperture is reduced to the size of the condenser's phase ring, the outstanding balance of contrast and resolution beautifully reveals transparent cellular structures. However, greater depth of field and the "halo" affect render it less than optimal in situations where large or thick objects are observed.

In 1978, Dr. Robert Hoffman patented an oblique lighting technique that is currently marketed under the name of Hoffman Modulation (Modulation Optics, Greenvale, NY). As you can see from Figure 3, Hoffman modulation requires the addition of an off-axis slit aperture in the condenser and a corresponding size bar shaped "modulator" in the objective, which has differing zones of light transmission (3%, 15%, and 100%). Both the

direct oblique source illumination and diffracted light are collected by the objective. The source light is suppressed by its passage through the 15% zone of the objective modulator, greatly suppressing its contribution to the image. One side band of diffracted light passes freely through the 100%, the other side band is suppressed by the 3% transmission zone of the objective modulator. This generates a contrast gradient (or shading) across the specimen. (The strength, or intensity, of the source illumination can be further varied by the presence of a continuously variable neutral density filter created by a polarizer crossed to a zone of the condensers' slit aperture which is also comprised of a polarizing material.) Halos, which are inherent in phase contrast, are eliminated in Hoffman by this linear gradient. If a cylindrical object (again, a thick cell) is viewed in Hoffman, the "lens" effect of the cell will cause the source illumination from one side to pass through the most suppressed area of the objective slit, yielding a darker image. However, the source illumination from the other side is refracted by the "lens" affect and is allowed to pass through the objective at full strength yielding nice shadows across the field. Similarly, 1 sideband of diffraction angles, generated by the specimen's structures, passes freely while the opposite band is suppressed. While this yields tremendous advantage in imaging thicker specimens, phase contrast continues to yield better contrast on very flat adherent cells, or fine cellular processes which extend from the cell (e.g., dendrites). Because its contrasting performance is unaffected by the birefringence of plastic chambers, Hoffman has been used in a variety of research and routine applications where plastic chambers are used. But because of the location of the objective slit aperture, if one also has applications requiring phase contrast, a second objective must be used. And due to the linear organization of the condenser and slit aperture of the objective, the alignment of the "system" is quite critical, and its light efficiency is poor.

### Variable relief contrast

In 1991, Carl Zeiss patented the principle of VAREL, which has been implemented into the Axiovert 25 inverted routine microscope, and, most recently, into the just-introduced Axiovert 200 & Axiovert 200 MOT inverted research microscopes.

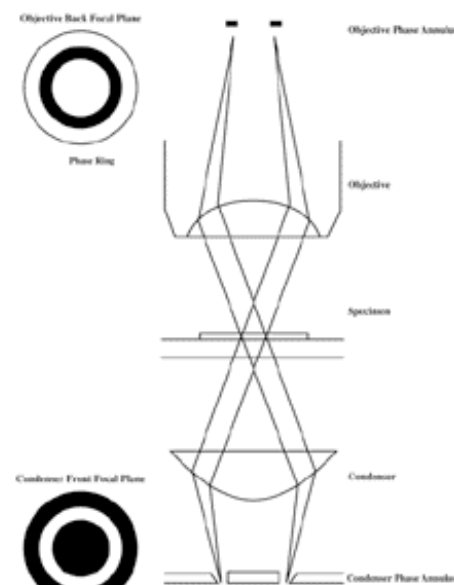


FIGURE #2 Phase Contrast

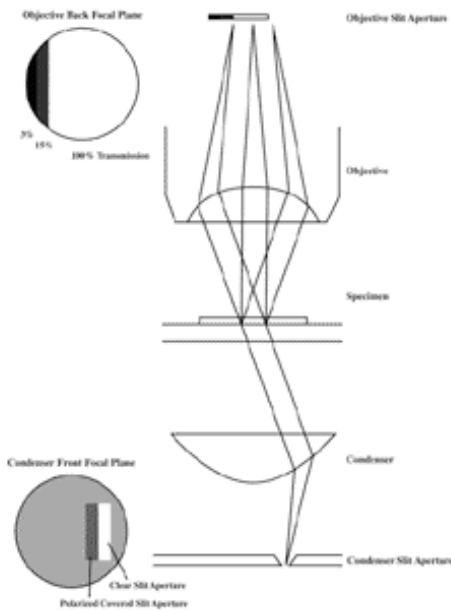


FIGURE #3 Hoffman Modulation

The principle is shown in Figure 4. You will notice from the objective cross section that a modified phase objective is used, allowing the same objective to be used in traditional phase contrast. When VAREL is needed, the condenser is moved to the VAREL positions and a segment of a matching annulus is moved into position. This slit directs the source illumination to pass through a second ring of high absorption (90+%) on the periphery of the objective aperture, suppressing its intensity by the density of that outer ring. The diffracted orders of light pass through the objective without being suppressed by the VAREL ring. The user also has the ability to manipulate the position of the slit, and

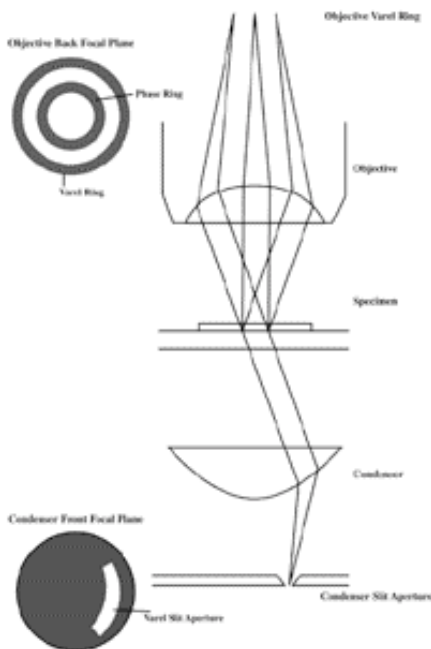


FIGURE #4 Varel Contrast

hence continually vary to what extent the source illumination is suppressed. Due to the concentric characteristic of that ring, the slit can be positioned from either side, allowing the shadowing affect we discussed with Hoffman, to be reversed. While initially only 10x and 20x objectives were introduced for VAREL, a 32x and 40x have followed.

The advantages of VAREL are clear. By the concentric nature of the slit, orientation is easy to achieve and maintain. And because of the location of the VAREL ring in the objective, Varel and phase contrast “rings” can be combined in the same objective. This provides a low cost solution to the plaguing problem of imaging in both traditional and routine tissue culture applications, to easily image a range of structures with varying “optical thickness”.

### Nomarski differential interference contrast

Our discussion of transmitted illumination techniques would be incomplete if we neglected to mention Nomarski Differential Interference Contrast, although its use is primarily found in demanding research applications to look at unstained cells and tissue. Because the basis of this technique is polarized light, it is restricted to specimen chambers which are not birefringent. This excludes most plastic chambers commonly used in tissue culture environments. Although the details of the optical principals of DIC are quite complex, the basic premise is as follows. As you can see from Figure 5, the DIC microscope has 4 basic components: polarizer, lower Wollaston prism, upper Wollaston prism, and analyzer. The polarizer restricts the illumination passing to the 1st Wollaston so that only linearly polarized light passes. The Wollaston prism splits the light beam into 2 polarized beams separated by a distance that is, in fact, below the resolution of the microscope. These 2 beams pass through adjacent areas of the specimen and are affected differently by its optical path differences. The 2nd Wollaston prism positioned above the sample recombines the 2 beams and the analyzer brings them to a common vibration direction, allowing them to interfere. The result is an image of contrast differentials based upon local differences of optical path length, as opposed to phase contrast, which looks at absolute optical path length. The advantage of Nomarski in research applications is its high resolution, by using the full NA of the

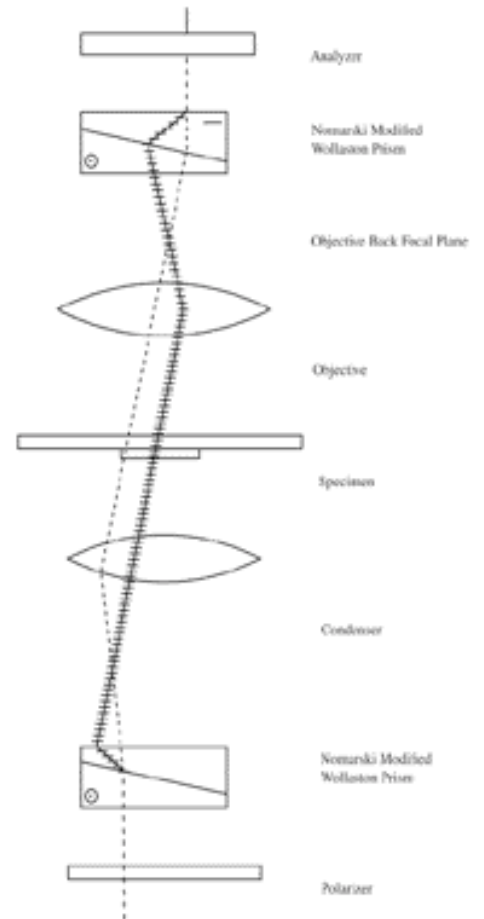


FIGURE #5 Normarski: Differential Interference Contrast (DIC)

condenser, and its shallow depth of field, giving one the ability to optically section through a sample. Its restriction to glass (nonbirefringent) specimen chambers and its inherent high cost, limit its use to demanding research applications.

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