OBLIQUE ILLUMINATION WITH PHASE CONTRAST MICROSCOPY

The illustration in Fig. 1 represents a photomicrograph of a thin section of decalcified bone taken in phase contrast with a Py 10x objective. The total magnification of the print is 150x. High contrast copy film was used.

Generally, it is very important to properly center the phase contrast condenser with respect to the optical axis and with respect to the annulus in the phase objective. Phase units are usually provided with a focusing telescope which serves to facilitate the matching of the phase rings in the illuminating and observation systems.

Fig. 1

The three-dimensional appearance of this particular illustration shows what can be done, if the condenser is laterally decentered. Oblique phase illumination produces an asymmetric diffraction pattern with very high contrast. The interpretation of such images must, however, be done with great care, because, if the path differences are arbitrarily "overextended", artifacts such as double contours may be observed which really are not representative of the material under observation.
MICRO-FLASH FOR PHOTOMICROGRAPHY

The use of electronic flash units for general photography including news and sports photography is well known. Its application in microscopy is becoming equally important, particularly when attempting to photograph living materials under the microscope. The extremely high intensity of the electronic flash coupled with its short exposure time will stop even the fastest motion.

Figures 2-5 are examples of photomicrographs taken with a phase contrast microscope coupled with the micro-flash unit illustrated in diagram Fig. 6. This flash attachment can be used in connection with all Leitz microscopes which have an in-base illuminator. The photographs were taken with a 40 x Phaco objective at an interval of approximately 8 seconds between each shot. The electronic flash unit requires approximately 8 seconds to recycle. The actual exposure time is calculated to be 1/1000 sec., thus making it possible to stop even the motion of the flagellum. This movement is so fast that the eye cannot clearly observe it.

Fig. 6

Most photomicrographic cameras, such as the Micro Ibso Attachment for 35 mm film with the LEICA body, are quite satisfactory for flash photomicrography, as long as the intervals between exposures can be longer than 8 seconds. The manual winding of the shutter, the transportation of the film and other manipulations require a certain amount of time before the camera becomes ready for the next picture. Ideally, photomicrography of moving objects should be carried out
with a fully automatic camera such as the Orthomat. The shutter release and film advance mechanisms are coupled, and pressing a button takes care of both operations automatically.

The light output of a flash unit is relatively constant; therefore, the exposure time would be the same for all pictures taken with the micro-flash unit. However, variables such as the absorption of the specimen, the speed of the film used and the magnification of the microscope are factors which require changes in exposure time. To arrive at a properly exposed negative the intensity of the flash must be corrected by means of a set of neutral density filters to suit specific conditions.
MICROSCOPE RESOLUTION

The question of maximum resolution possible for any microscopic image will always be one of the important problems of microscopic theories and techniques. The aperture of the cone of transmitted light and the correction of the condenser are two of the factors which affect the attainable resolution. The effect of the condenser aperture upon image resolution is given much attention; but, usually, the degree of correction of the condenser is not clearly expressed in either theoretical or practical terms.

However, in practical microscopy, the state of correction of the condenser to obtain adequate image contrast is very important. Without this correction -- even if theoretically resolvable -- the structure (based upon aperture consideration) alone remains invisible. The fact is that the image contrast is directly affected by the correction of the condenser.

We are going to concern ourselves with the following:

a) When can high aperture condensers be utilized to full advantage?

b) What degree of condenser correction is required?

We will try to show that only a rather highly corrected condenser system permits the use of theoretically desirable condenser apertures. The substage condenser in a microscope for transmitted light -- according to the principle of Koehler illumination -- performs a two-fold function:

First, it transilluminates the specimen with parallel light in a cone of high aperture. This can be seen in Figures 7a and 7b.

Fig. 7a

Fig. 7b
Fig. 7a shows a pencil of transilluminating light originating from the front focal plane of the condenser parallel to the optical axis.

Fig. 7b shows a parallel pencil of transilluminating light which originates at the periphery of the aperture stop. Although this pencil of light passes through the specimen at a high angle of incidence, it is parallel in itself. The sum total of all pencils of light originating from each area in the plane of the aperture diaphragm forms a cone of high aperture light. The front focal plane of the condenser is conjugate to the plane of the light source. An enlarged image of the light source is projected into this plane to serve as a substitute for the light source. Fig. 8 shows how this is achieved by means of a collector lens.

The second function of the condenser, according to the Koehler principle, is to project an image of the field diaphragm sharply into the specimen plane. This is shown in Fig. 9. The field iris is usually mounted at some distance from, and below the aperture stop. Its image restricts the illuminated specimen area; this eliminates glare and light scattered from areas not in the field of view. Correction and quality of this reduced field diaphragm image in the object plane have a profound influence upon the final quality and resolution.

The aperture stop regulates the angle of the cone of transilluminating light, or "the aperture of the illumination". It adjusts image contrast and resolution and should under no circumstances be used to regulate just light intensity.
The original formulation of the Abbe theory of image resolution assumed not a fully opened, but a rather narrowly closed aperture diaphragm; the theory explains that the image of two closely neigbored, transilluminated object points will show them as being two points, or being "visually resolved", when their distance is not less than

\[ d = \frac{\lambda}{nA_{\text{obj}}} \]

The two diffraction disks will then be separated by an intensity dip of approximately 20%, and this is visually required to recognize a separation.

Abbe also discussed the case of a narrowly closed, but highly decentered aperture stop. Under these conditions of transillumination with a high angle of incidence, or under high aperture, the same 20% intensity dip in the elementary image of the two close object points may be obtained when the two points are separated by only a distance of approximately

\[ d = \frac{\lambda}{2 \ nA_{\text{obj}}} \]

If one now transilluminates a specimen with a fully opened aperture stop, the intensities of all the elementary images will be superposed. Only the high aperture light, however, will produce an elementary image that resolves the fine detail under study.

Unfortunately the combined, superposed intensities of all the other, non-resolving, or structure revealing elementary images are so much stronger that in most cases it is not possible to maintain the required contrast in the resolving, elementary image. The fine detail in the object would have to have very high inherent contrast for this.

It is, therefore, rarely possible to reach the theoretical limit of resolution with such specimen as they occur in practice. The discussion of the effect of high aperture illumination, the need for, and the desirability of high condenser apertures shows quite clearly that, by definition, microscopic resolution is a function of the image contrast attained. In brightfield microscopy this is naturally lower than the object contrast; the contrast loss being determined by the contrast transfer capabilities of the optical system of the microscope.
The equation usually given for microscopic resolution

\[ d = \frac{1.22 \lambda}{2 n A \text{ obj.}} \]

implies such a high degree of object contrast that the latter is not the limiting factor. Most biological preparations, however, do by far not even approach such high inherent contrasts. It is, therefore, in practical microscopy almost always necessary to close the aperture stop to some extent, so that the image attains sufficient contrast. One will rarely work with illuminating apertures of much more than 0.9, even when an immersed condenser top element is employed. High condenser aperture is, therefore, not necessarily of such advantage in routine work as is occasionally assumed. The demand for condensers with high apertures is probably to be explained by the fact that high aperture condensers usually were the best corrected systems. The optical correction of the condenser has a very substantial effect upon what is generally described as "image crispness, clarity, or definition". These are not the terms that one would use in quantitative analysis of the system, but they convey the microscopist's direct visual impression. The condenser correction directly affects image contrast, and with this, the attainable resolution especially when Koehler illumination is employed. An ideally corrected condenser should project a sharp, reduced image of the field stop into the object plane; and it should transilluminate the object with strictly parallel pencils of light up to high apertures. This requires the following corrective measures:

- spherical correction,
- chromatic correction,
- correction for chromatic difference of spherical aberration (Gauss-aberration),
- fulfillment of the sine-condition.

Systems in which the chromatic difference of the spherical aberration is eliminated, and which fulfill the sine-condition are called "aplanatic systems". Spherical correction of a condenser is the minimum requirement. Without it the different lens zones of the condenser produce images of the field diaphragm plane at different focal distances; the high aperture rays form an image much closer to the condenser than the central rays. The result is an indistinct image of the field diaphragm, and low contrast in the final image of the specimen. It is not recommended to use a spherically uncorrected condenser for photomicrography. Condensers with an a spherical correction only may be used for b/w photomicrography, provided that a strict monochromatic green filter is employed.
Chromatic correction is necessary for a condenser used in color photomicrography. Chromatic aberration produces not one, but a sequence of colored images of the field diaphragm plane, one behind the other at different focal distances from the condenser. But even achromatic condensers are rather sensitive with respect to improper focusing. A condenser position only a few hundred microns below or above the prescribed setting will—in color photomicrography—invariably produce a strong color hue in the background, and a definite lack of image definition. The apparent shift in color temperature due to incorrect condenser focus is much more pronounced than usually results from an incorrect setting of the light source transformer. Too high a condenser focus causes a bluish, too low a focus a yellow cast in the background.

The best systems are the achromatic-aplanatic condensers. Systems of this type have spherical aberrations corrected not only for one color but for a wider range of the spectrum; simultaneously the sine-condition is fulfilled. The sine-condition in short demands the following:

The condenser projects a reduced image of the field diaphragm into the object plane. In a spherically corrected system the different lens zones will all project this image into the same focal distance. The different lens zones may, however, produce images with different lateral magnifications, or as it would be the case here, different lateral reduction ratios. The result is an indistinct image. When the sine-condition is fulfilled, all lens zones in the condenser, the high aperture zones as well as the central ones, produce a field diaphragm image with one and the same reduction ratio.

In the discussion of desirable condenser qualities and specifications the question of condenser aperture receives great attention. Practical experience as well as theoretical considerations, however, have shown that the degree of condenser correction has a decisive influence upon image quality and attainable resolution. A high degree of condenser correction, therefore, is the prerequisite of the use of high condenser apertures, and should by all means be a primary consideration.