REICHERT

THE PRINCIPLE OF PHASE CONTRAST MICROSCOPY

In order to obtain a clear microscopical image in which the finest details are easily discernible using ordinary brightfield microscopy, the object must possess differences in absorption (amplitude changes of the light waves). The frustules of diatoms, which are composed of silicon dioxide, are clearly visible on their own without any additional artificial aids. Most biological specimens, however, only show refractive index changes from one object detail to the next. To make these visible one would have to stop down the aperture iris diaphragm under Koehler Illumination to the point of impairing the resolution. Such biological specimens are, therefore, fixed and stained to artificially produce differences in absorption (differences in opacity). Fixing and staining, however, kills the specimen and might on occasion even substantially change it.

Every examination of u n s t a i n e d materials presents difficulties which cannot be solved satisfactorily by any one of the usual methods:

Illumination with a wide cone of rays ensures good resolution, but fine details will be lost and even the best resolution is rendered useless if the resolved details lack contrast and, therefore, remain invisible.

Illumination with a narrow cone of rays (too low a numerical aperture) considerably enhances the contrast, but the resolution is lost and the appearance of so-called "diffraction fringes" produces a poor and untrue image.

Darkfield illumination can only be used for objects which, compared with the field of view, are relatively small and it, therefore, fails when more or less flat objects are to be observed since it reveals the contours rather than the internal details.

There are, however, many instances when staining is out of the question, particularly when a biological object is to be examined in its completely unchanged natural state and, if practical and desirable, even in its living state or in its natural surroundings (secretions etc.) .

The physicist F. Zernike has shown that there is a method by which clear and well defined contrasty images of u n s t a i n e d objects with only very small differences in refractive index can be obtained while fully retaining true optical representation at good resolution. This method is called Phase Contrast Microscopy. Its principle, which is based on more complicated mathematical calculations than can be given here, may be described briefly as follows:

When light strikes an object consisting of a fine grating, some light passes unchanged in its direction (the direct light or zero order spectrum) and some light is diffracted at the edges of the specimen details to form diffracted beams of the first to the n^{th} orders in a similar way in which a prism disperses a beam of white light into its component colors (one spectrum). Most of these rays are imaged at the back focal plane of the objective where they may be observed as a series of spectral image maxima, forming a rather complicated light figure, the so-called "diffraction pattern". The higher the numerical aperture of the objective, the more image maxima will enter into the image forming train of rays. The quality of this "diffraction pattern" also depends just as much on the type of illumination used and, most important of all, upon the structure and the nature of the object itself.

If, on the other hand, the structure of the object be regarded as composed of strips of transparent material having slightly differing refractive indices (e.g. small living organisms formed of transparent tissue immersed in an aqueous medium) then rays which have passed through these alternate strips acquire mainly phase differences (differences in the speed of light passing through difference parts of the specimen). The human eye, however, is not equipped to recognize such phase differences. It can only see differences of amplitude of light rays, i.e. differences in brightness. The main problem, therefore, was to look for an expedient which might be able to convert the invisible phase differences into visible amplitude differences.

In the phase contrast method the diffraction pattern is subjected to interference. This interference effects a definite alteration in accordance with certain laws of the mutual relationship between the oscillating phase of the diffracted and the undiffracted light. Additionally, the amplitude of the light which forms the zero maximum is reduced to a suitable degree by passage through a neutral grey filter. The change in relationship of the

phase as well as the reduction in the amplitude is brought about by specially provided phase and absorption altering elements, commonly referred to as phase plates. They are made by the deposition of films of predetermined thickness by high vacuum thermal evaporation processes. Annular patterns of this deposition have been found to be most practical and effective as they introduce practically no undesirable lack of symmetry into the image on account of their own complete axial symmetry. The size of the phase annulus is dependent upon the numerical aperture of the particular objective.

As already stated, this phase plate is introduced into the back focal plane of the objective and covers a definite numerical aperture range of the entire numerical aperture of the objective.

As the back focal plane of every type of objective lies in a different position and in most objectives even within the lens system, it is obvious that one and the same phase annulus cannot simply be introduced into all objectives. Specially computed CONTRAST OBJECTIVES are required. These objectives are fitted with phase annuli particular to the type of objective before they leave the factory.

A special PHASE CONTRAST CONDENSER is also required. Every maximum in the diffraction pattern is, in form, an image of the aperture diaphragm of the microscope condenser. If the opening of this diaphragm is in the form of a circular area, as is the case with all normal microscopes, the individual maxima in the back focal plane will overlap so much that it will be impossible to limit the necessary modification to the zero maximum only. To reduce the amount of overlap of the individual maxima, the aperture diaphragm of the phase contrast condenser is made in the form of a light transmitting annulus. The phase contrast condenser with a numerical aperture of 0.95 has, therefore, only a single lens system; but it is equipped with a number of annular aperture diaphragms matched in size exactly to the phase annuli of the phase contrast objectives being offered.

To ensure perfect working, it is finally necessary that the image of the annular aperture diaphragm of the phase contrast condenser should be exactly centered to the phase annulus of the phase contrast objective so that the direct light from the phase contrast condenser will go through the annulus of the phase contrast objective. A simple AUXILIARY TELESCOPE with a focusing eyepiece is, therefore, being provided. For the initial setting of the illumination it is inserted into the microscope tube in place of the regular eyepiece. The auxiliary telescope focuses on the back focal plane of the microscope objective so that the annular illumination coming from the phase contrast condenser can be adjusted concentrically to the annulus of the phase contrast objective.

MICROSCOPIC OBSERVATIONS WITH ANOPTRAL CONTRAST*

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Microscopes have been in use for more than a hundred years, and they reproduce very well the structure of objects which are fixed and stained and usually embedded in Canada Balsam. However, there are certainly many unknown fundamental facts that can only be revealed by studying the living preparation directly. Life is sometimes characterized much better by motion, growth, propagation and exchange of the cellular constituents than it is by the multiplicity of structures. There are, however, difficulties in the way of studying unstained preparations.

All those who have worked with the microscope must be aware of the fact that a normal stained biological preparation provides an excellent picture when viewed through a microscope with large objective and condenser apertures, whereas in the absence of staining very little if anything can be seen since the details are only distinguishable then by their refractive power. Not until the illuminating aperture has been reduced, e.g. by stopping down the iris diaphragm, do the details begin to appear one after another, but even then they are surrounded by concentric diffraction fringes which have a detrimental effect in that they reduce the resolving power.

Nor does darkfield microscopy help very much. Abrupt changes in the refracting properties of the details of the object certainly show up clearly, but small differences or gradual changes in the refractive index remain invisible.

These drawbacks led the author to undertake extensive experiments with the "Schlieren" method discovered by Toepler nearly a hundred years ago, and to apply them to microscopy. It was soon discovered that an annularly

* ANOPTRAL CONTRAST is a special type of Contrast resembling negative Phase Contrast - with a pleasing, restful brownish background and WITHOUT HALOES.

defined aperture in both the condenser and the objective was the most suitable one. The realization of annular defined apertures in objectives, however, was very difficult. After experimenting with thin coatings of silver, without much success, the author tried coatings of soot applied by repeatedly exposing the surfaces of an objective lens to a candle flame.

It was not within the author's methods to go more deeply into the optical laws on which the effects were based. What did really matter was that a microscope modified on the above lines provided much better images of living specimens viewed through it. The obvious course, then, was to proceed on purely empirical lines. It was soon evident that excellent results could be obtained by using an annular diaphragm opening in both the condenser and the objective, the transmission of the objective diaphragm being 50 per cent. Living specimens such as yeast cells appeared very clear and sharply defined with this system, and had an agreeable brownish tint on a bright background. The cells showed darker the more the refractive power of their content exceeded that of their surroundings, i.e. the better their state of nourishment.

At that time the second world war made communication between Finland and other countries difficult; it was not until much later that the author found that the phase-contrast microscope, developed on a theoretical basis by Zernike, had been constructed. When the author looked into a phase-contrast microscope for the first time he was surprised to note the similarity of the images obtained with it and with his own equipment. Both of them certainly had annular condenser apertures, but the phase-contrast objectives had light-absorbing annuli whereas the author's had an absorbent coating recessed in the form of a ring. But the effect produced by the images was not identical. Round each light-refracting detail the phase-contrast microscope exhibited haloes which had no counterpart in reality. The author's microscope showed none of these haloes, and the graduation of the different intensities of contrast, depending on their light-refracting properties, was agreeably soft. On the recommendation of certain experts, the Swedish Jungner Company offered their help in manufacturing the equipment; but the method, which the author originally termed the "umbral method", was shelved at first because of patent difficulties. However, the author would like to express his gratitude to the Jungner Company for their kindness and help.

During his first experiments the author made an observation which was afterwards to prove significant. If the coating of soot in the objective was not recessed in the form of an annulus, but complementarily applied so that the light coming from the condenser diaphragm passed through this annulus, an image was obtained which exhibited the opposite properties. The author resumed his experiments in this direction in 1952, partly because the available phase-contrast microscopes had not been developed further. The halo effect in them, mentioned above, continued to prove disturbing and often led to false interpretations.

When attempts were being made to improve this second method it became clear that increasing the light absorption of the soot ring to over 90 per cent gave a particularly well contrasted image with a golden-brown background against which the details of the object usually appeared bright (because of their higher optical density), but were surrounded by narrow darker zones. The image was, so to speak, the reverse of an ordinary phase-contrast image, with dark borders instead of the bright haloes. The golden-brown tint of the image was a happy chance, for it is agreeable and restful to the eye - a fact long known in art photography. The question is now: are the dark shaded zones of the picture less disturbing than the bright haloes of ordinary phase-contrast microscopy?

The answer is a definite affirmative. We recognize the shape of most objects better if we see them lighted as they usually appear in our everyday surroundings. The objects are not as a rule presented in transmitted light, or as silhouettes, but appear in our visual field illuminated mostly from above, from the side, or from several directions at the same time. The diffuse light from the sky is probably the most original and natural form for the human eye. If we look at a stone on sandy ground under this mode of illumination, we can see a lightly shaded zone around it. If we saw the same thing as a negative, in which the half-shadow borders were replaced by haloes, it would be difficult to apprehend the shape of the object.

The normal image of the stone on the sandy ground bears the same relation to the negative as the microscopic image produced by the author's second method has to the image obtained by "positive" phase-contrast. The disturbing haloes - simulating phosphorescent edges - of the latter, are converted into a virtue by their reversal, since this half-shadow bordering assists the apperceptive faculty and produces an almost plastic effect.

The writer has also been able to familiarize himself with the "negative" phase-contrast objectives of different makes. The image effects obtainable with them are, of course, somewhat similar to those obtained by the

author's second method, but the pictures give rather a cold impression because of their greyish-white color accompanied by a metallic sheen and a strange fogginess. As the phase annuli are made by vacuum evaporization and especially as metals are used, the light-absorbing layers tend to introduce reflecting surfaces into the system. Since the degree of absorption of the phase annuli myst be considerable in order to obtain a very high degree of contrast, it is no wonder the stray light from these reflections becomes nearly equal in brightness to the image itself. The soot surface, on the contrary, reflects very little. For the commercial production of the new objectives, the coating of soot, as it is not very resistent, must be replaced by other suitable substances which produce the same effect. Messrs. Optische Werke C. Reichert A.G., Vienna, Austria, have solved this problem by using means available only to a large firm of microscope makers. They are now manufacturing these objectives and their accessories on a commercial basis under the name of "Anoptral Contrast Equipment" (anoptral = non reflecting). One important point in this connection was how to obtain the beautiful golden-brown tint provided by the layer of soot and which is necessary to obtain the desired results. However, Messrs. Reichert have succeeded in solving this particular problem completely satisfactorily.

SUMMARY

Through the use of annularly defined zones of special coating instead of conventional phase rings and by making the ring apertures larger than usual, much better contrast and resolving power can be obtained than by using the ordinary phase contrast.

In Anoptral Contrast as produced by Optische Werke C. Reichert A.G. of Vienna, the transparency of the coated ring is particularly small. The image becomes "negative" in the ordinary sense; physiologically, however, it is much more natural than the "positive" one because the haloes unavoidable in the latter are converted into shadow borders giving the image an illusion of depth. In addition to this, the gold-brown tint of the anoptral image is very agreeable to the eye.

LITERATURE

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