Nomarski Differential Interference-Contrast Microscopy

Part IV: Applications

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In the comprehensive description of Nomarski differential interference-contrast (DIC) microscopy, the fundamentals and experimental designs (Part I) and the formation of the interference image (Part II) were discussed and a comparison made with the phase-contrast technique (Part III) [26, 27, 28]. The present Part IV will now give a review of the applications of Nomarski DIC microscopy together with a list of references. No claim is made with regard to the completeness of the bibliographic data in view of the rapidly increasing number of applications and publications on the subject. The bibliography is therefore only intended to serve as a guide to the potential uses of the Nomarski DIC method in the different fields of microscopy of organic and inorganic objects.

A. Microscopy of organic objects

An essential advantage of Nomarski DIC microscopy is the fact that – like phase contrast, for example – it allows the examination of unstained specimens. A new and extremely useful aid has thus been created, above all for examining living specimens under the optical microscope.

Cytology

Using a living cell of Haemanthus katherinae in the process of division as an example, *Bajér* and *Allen* [5] demonstrate the superiority of the DIC image over phase-contrast representation: while in phase contrast the halo effect makes it impossible to recognize details, the spindle fibers can be clearly seen by the Nomarski differential interference-contrast method.

DIC micrographs of hela cells in a nutrient solution and denatured with 96 % alcohol were published by *Gabler* and *Herzog* [18, 19].

Wunderer and *Witte* [47] published a comparison of photomicrographs of cells and groups of cells from the mucuous membrane of the human stomach, taken by phase contrast and by Nomarski differential interference contrast. These examples prove that the two methods complement each other very nicely. However, in the case of a group of glandular cells of the gastric mucosa, the interference method offers the advantage of improved detail definition.

Giving many practical examples, *Padawer* [39] explains the characteristics and advantages of DIC microscopy. In one particular example nuclei and vacuoles appear in the DIC image as depressions, while highly refractive structures such as eosinophile granula or fatty inclusions seem considerably elevated. Also the vacuoles of macrophages appear as depressions, while the nuclear membrane shows up as a bulge. The author shows that phase structures located outside the focal plane cannot always be neglected in the interpretation of DIC images. When erythrocytes are viewed by phase contrast, the formation of haloes is rather troublesome. In this case, the DIC image is unmistakably superior. The situation is similar with epithelial cells of the mucous membrane of the human mouth. *Duitschaever* [14] uses the DIC method for microscopic investigations on somatic cells in cow's milk and other body fluids. *Engels* and *Ribbert* [15] also use Nomarski differential interference contrast for the examination of nucleoli in Musca domestica. *Ribbert* and *Bier* [41] make use of the Nomarski method for studying insect ovaries.

Stoll * and *Gundlach* [43] compare the phase-contrast image with the DIC image of a cell smear in a saline solution. The living trichomonad beside an epithelial cell and erythrocytes shows more detail in interference contrast. This holds true above all for the marginal portions of the trichomonad which in phase contrast reveal considerable flare due to halation. A similar situation is encountered in the cell smear in a saline solution. This example also shows that it is easier to distinguish superimposed structures in the DIC image than in phase contrast. The authors prove that in such a case it is frequently impossible to recognize the borders of the cell in phase contrast due to halation.

Botany

The phase-contrast technique is well suited for examining small particles – especially organelles – in protoplasm [44]. However, the great depth of field of this method is a disadvantage in botany. As a result, phase structures in the light path will impair the phase image even if they are located outside the focal plane [44]. According to *Url* and *Gabler* [44], the shallower depth of field of Nomarski DIC microscopy opens up a considerably wider field of application for light microscopy in botany. These authors show, among others, DIC micrographs of the inside and outside epidermis of Allium cepa, cells of Closterium lunula and – in a comparison with phase contrast – Micrasterias denticulata and Closterium lunula. In the case of Allium cepa, mitochondria, the Golgi complex, leucoplasts, the nucleolus and large and small spherosomes stand out in high contrast, the latter due precisely to the great difference between their own refractive index and that of the surrounding areas.

According to *Padawer* [39], observation of plant material offers considerable difficulty, be it in phase contrast or differential interference contrast. In the one case, the pronounced difference of refractive index gives rise to heavy halation, in the other birefringent components disturb the image. This has been proved for example in the case of dried pollen, such as Salix discolor and above all Coreopsis. Similar conditions are encountered with freshwater Chlorophyceae. *Maguire* [29] investigates subchromatid structures in corn with the aid of the Nomarski method and, for comparison, in phase contrast.

Using the African blood lily, Haemanthus katharinae, as an example, *Allen*, *David* and *Nomarski* [3] show that the spindle fibers of a living cell during division stand out clearly in the DIC image (see also [5] and [6]), whereas they are hardly visible by any other microscopic techniques. *Baum* [8] uses DIC microscopy to show natural hybrids of Avena sativa and Avena fatua in the cultivated oat.

Histology

Gabler and *Herzog* [18, 19] show the thyroid gland of a mouse in positive and negative phase contrast as well as in Nomarski differential interference contrast. Nomarski DIC is also suited for amplitude staining of stained specimens, as shown by *Allen*, *David* and *Nomarski* [3] on large chromosomes of Drosophila melanogaster. Even human chromosomes can easily be examined by the DIC method using amplitude staining. Very thick bone sections generally used, for example, for examination by incident fluorescent illumination, result in a noticeable decrease in contrast in the DIC image, same as in phase contrast. This is due to the fact that proper imaging of the contrast-producing components, such as annular diaphragm on phase plate or auxiliary prism on principal prism, is no longer guaranteed. This is demonstrated by *Lang* [28] both for phase contrast and Nomarski differential interference contrast on the example of an excessively thick transparent specimen (polished bone section) and a thin, well-suited transparent specimen (rat's tongue, unstained). Despite this qualification, the Nomarski DIC method, owing to its high useful aperture and the consequent small depth of field, is the ideal method for the observation of so-called optical sections. This may be verified by an example from zoology: Fig. 1 shows photomicrographs of Macronyssus bacoti in bright-field (a), phase contrast (b) and Nomarski differential interference contrast (c) all with the same focal

* See the monograph meanwhile published by Peter Stoll: Gynecological Vital Cytology, Springer-Verlag Berlin, Heidelberg, New York 1969, which contains numerous practical examples of DIC microscopy, often with comparative phase-contrast micrographs.

plane. Figs. d and e also show the DIC image of the same object with two other focal plane settings.
The Nomarski DIC method makes it considerably easier to analyse the structure of thin sections as is shown by the contrast with the bright-field view in Fig. 2. For this type of work the DIC method can also be successfully applied with stained specimens. The colour distortion caused by the Nomarski method in such cases remains within reasonable limits. It should also be remembered that the transition to bright-field observation for comparison purposes, for instance by removing the interference contrast slide from the light path, is swift and convenient.

Hematology

With the aid of Nomarski DIC microscopy, unstained erythrocytes can be rendered visible with excellent results (*Gabler* and *Herzog* [18, 19]). According to the authors, the DIC image of a crystal in the blood lymph of an eel is superior to the corresponding phase-contrast image that is impaired by halation.
Padawer [39] discusses differences between phase-contrast and differential interference-contrast observation of the hemolysis of frog erythrocytes. The photomicrographs taken under identical conditions show non-hemolyzed cells, spherocytes and completely hemolyzed cells. The author shows that with normal cells the nucleus in the DIC image stands out more clearly from the cytoplasm than in phase contrast. The nucleus becomes more elevated from the cytoplasm all the more clearly the more water the cell absorbs and the more hemoglobin it loses. With completely hemolyzed cells the cytoplasm will show up only weakly due to the loss of hemoglobin, while the nucleus stands out in good contrast. In another case, viz. a fresh blood smear, the coiling makes phase-contrast observation impossible due to heavy halation. In the DIC image, however, sufficient detail can be recognized in spite of the stratification. In phase contrast, fibrin fibers may appear dark or bright, depending on whether or not they lie in the focal plane. This complication does not exist in differential interference contrast.

Neurology

Neuhoff [31] uses Nomarski DIC microscopy to render human ganglion cells visible and especially for examining cells in which an appendix leads back to the same cell, so-called feedback neurons.

Bacteriology

With bacteria specimens, the disturbing halation known from phase-contrast images presents an advantage in Nomarski DIC microscopy; as an example, *Gabler* and *Herzog* [18, 19] show a smear of Klebsiella.

Hydrobiology

Quite a number of authors have published DIC photomicrographs of diatoms which show up 3-dimensionally in the DIC image. Due to the excellent resolution of the Nomarski DIC method, minute detail can be recognized in the diatoms. *Gabler* and *Herzog* [18, 19] show the DIC image of Auliscus sculptus. The use of Nomarski DIC microscopy in micropaleontology is described by *Barbieri* and *Mazzola* [7].
Padawer [39] compares phase-contrast and differential interference-contrast images of various diatoms. In this comparison, the superiority of the DIC image is very evident. In a very comprehensive paper, *Allen, David, Hirsch* and *Watters* [2, 13] cover the subject of image interpretation in transmitted-light polarizing interference microscopes both of the image-duplication and the differential type. In addition to an extensive comparative discussion of theoretical and experimental principles, the differences are illustrated impressively by a number of practical examples. Under Nomarski even large path differences of up to $2\,^1/_4\ \lambda$ between Stauroneis acuta diatoms and the mounting medium give images that are rich in detail. The Surirella robusta diatom can be reproduced with good contrast even with an illuminating aperture of 1.25. As a result, object details that are invisible at a smaller numerical aperture of, for example, 0.6, can be clearly distinguished.
Allen, David and *Nomarski* [3] report on the fundamentals, design, function and characteristics of ZEISS differential interference-contrast equipment. A number of practical examples explaining the special features of the equipment concern diatoms: Staureonis acuta diatoms in the DIC image as compared to the interference-contrast image (photographed with the ZEISS Jamin-Lebedeff system) show that particularly pronounced gradients of optical thickness in the specimen are reproduced very clearly in the DIC image. The azimuth effect of the technique can be demonstrated very impressively in the Hantzschia amphioxys diatom. Radial structures such as Anachnodicus ehrenbergii diatoms also reveal the azimuth effect. Using the example of the Surirella robusta diatom, the authors explain the advantage of DIC equipment over bright-field and phase-contrast observation, in that excellent contrast is obtained even at high aperture. In other words, the Nomarski DIC method has the effect of a filter that amplifies high spatial frequencies and subdues low ones. The advantage of the shallow depth of field of the DIC method as compared to phase contrast is illustrated by a Triceratium favus diatom.

B. Microscopy of inorganic objects

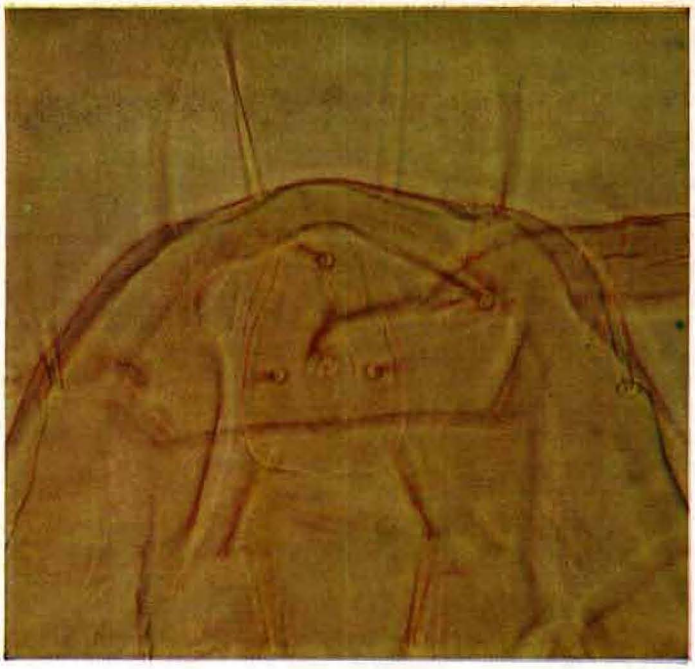
Metallography

As early as 1954, *Nomarski* and *Mme Weill* [32] pointed out the advantages of differential interference-contrast microscopy in the field of metallography (e. g., electropolished cobalt). A second publication by the same authors [33] dealt exclusively with metallographic applications. Among other things, it was devoted to a detailed study of various growth spirals of silicon carbide (SiC). *Nomarski* and *Mme Weill* were able to prove that growth steps of 440 Å ± 30 Å for example, can be resolved without difficulty. Under certain conditions, a relief of the order of a few Angström units can be recognized in the DIC image. Slipbands in cobalt subjected to a tension of 440 g/mm² for a period of five minutes can be reproduced with excellent contrast. The photomicrographs show various patterns of slipbands which, with bright-field illumination, for instance, can be recognized only with difficulty or not at all.
Measuring thin films of an order of magnitude of 2000 Å with the aid of yellow sodium light (589 μm) and Nomarski differential interference equipment, *Le Méhauté* [24] obtains an accuracy of ± 1.5 %. The author comes to the conclusion that the DIC method is superior to bright-field and particularly to phase-contrast observation for testing highly polished surfaces, examining thin films on glass substrates (vacuum-deposited films), checking quenched steel for undesirable phases such as ferrites, austenites, etc., and finally for the testing of diffusion processes by phase changes, the creation of new phases, recrystallization or other defects such as porosity due to different rates of diffusion between two elements, and lastly checking for dislocations and displacement of grain boundaries. As practical examples *Le Méhauté* publishes photomicrographs of quenched Cr-Ni-Mo steel, cold-worked bronze and sintered iron.
Bertocci and *Noggle* [9] use a differential interference-contrast system for the quantitative examination of small etched copper surfaces down to a mean size of 6 μm. Depending on the magnification of the objective used, they attain an accuracy of between ± 5′ and ± 30′.
The superiority of Nomarski DIC microscopy over bright-field observation is illustrated by *Gahm* [20] who compares photomicrographs of unalloyed quenched and tempered steel taken by both techniques. The deformation of the surrounding area produced by a microhardness indenter, of which no trace is visible in bright field, can be clearly distinguished in the DIC image. At the same time, the series of micrographs shows the azimuth effect characteristic of the Nomarski DIC method, which can here be observed along a linear grinding trace. *Jeglitsch* and *Mitsche* [23] use Nomarski to investigate the metallographic structure of Vacutherm samples (Widmannstätten structures, ferrite after γ/α-conversion, pearlite containing 0.85 % C), of low-temperature martensite, white cast iron (hypereutectic and hardened) and fused high-speed steel. On the left, Fig. 3 shows a martensite needle magnified 800 x in bright field, on the right, the same needle in Nomarski differential interference contrast.

Crystallography

In 1954 *Nomarski* and *Mme Weill* [32] reported on numerous practical examples and illustrated the use of Nomarski DIC microscopy in crystallography, e. g. growth spirals on silicon carbide (SiC) with triangular

1a



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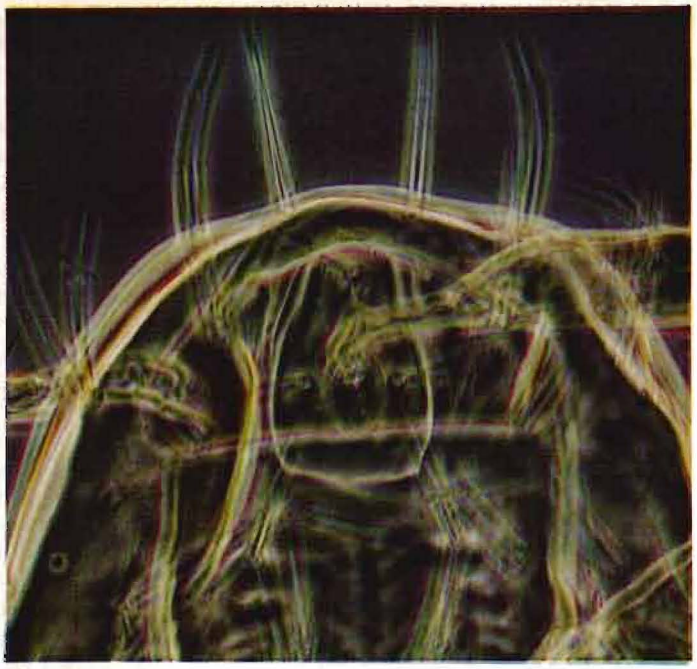

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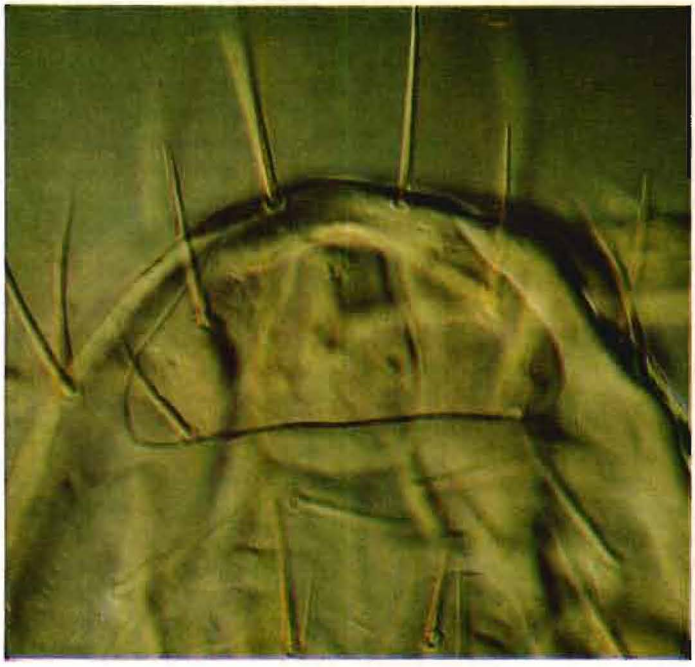

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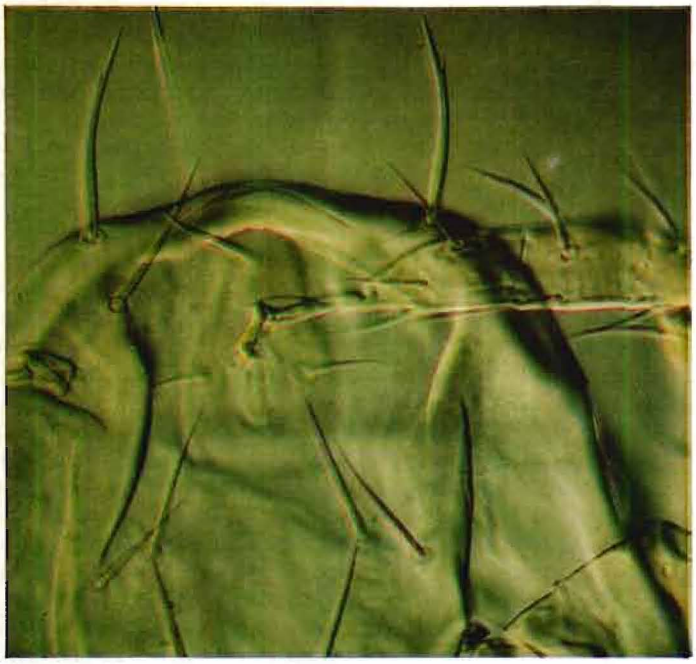

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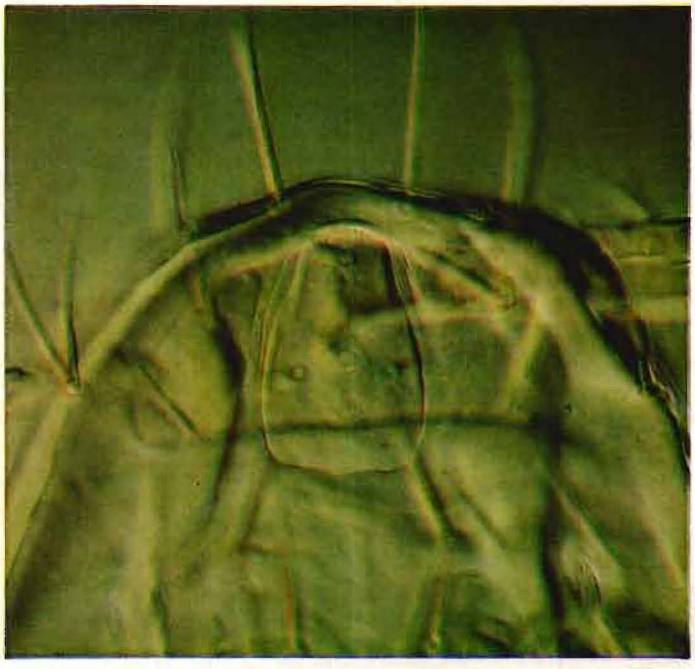
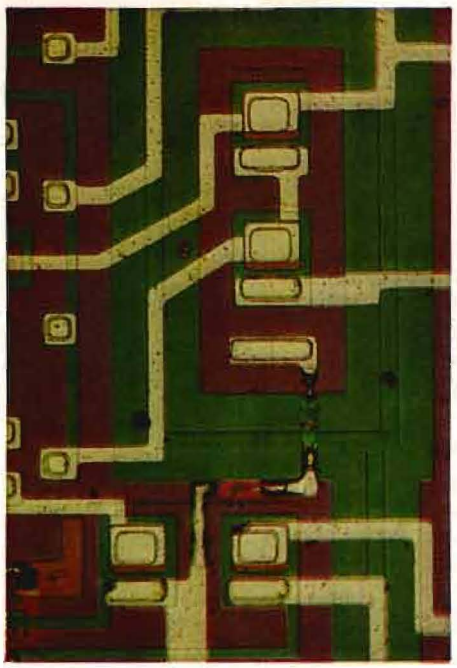


Fig. 1: Macronyssus bacoti in bright field (a), in phase contrast (b), and differential interference contrast (c, d, e). Figs. d and e were taken with different focal plane settings. ZEISS Ultraphot II, Planachromat 40 x, 0.65 N. A., magnification 320 x.

2a

2b

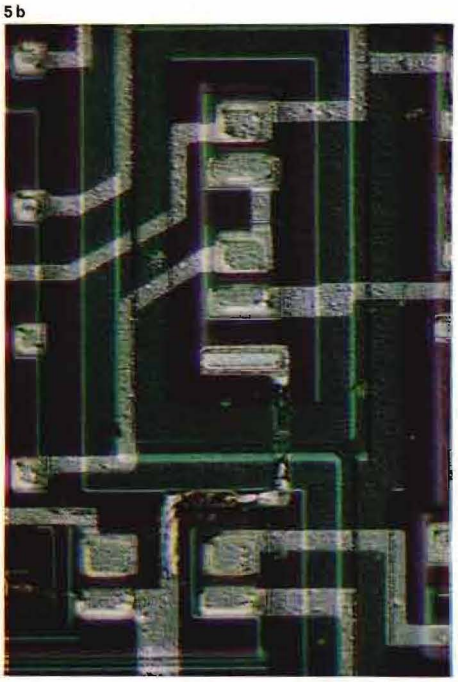
5a



3a

3b

5b



4a

4b

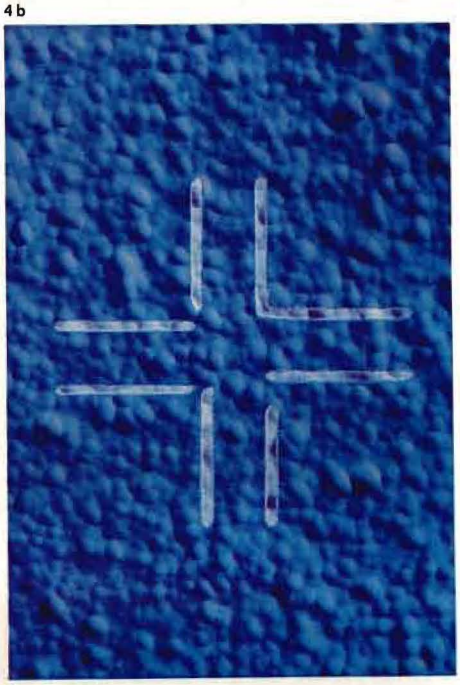


Fig. 2: Cat muscle in bright field (a) and in Nomarski DIC (b). ZEISS Ultraphot II, Planachromat 16 x, 0.35 N. A., magnification 144 x.

Fig. 3: Martensite needle, bright field (a), Nomarski DIC (b). ZEISS Ultraphot II, Epiplan 100 x, 1.25 N. A., oil, magnification 800 x.

Fig. 4: Wafer: bright field (left) and Nomarski DIC micrograph (right). ZEISS Ultraphot II, Epiplan 8 x, 0.2 N. A., magnification 72 x.

Fig. 5: Cross-section of an integrated circuit that failed due to overload, in bright field (a) and in Nomarski DIC (b). ZEISS Ultraphot II, Epiplan 8 x, 0.2 N. A., magnification 72.

symmetry; principal spirals with secondary recrystallization on SiC; star-shaped growth spirals etc.

Using the example of microhardness indentations in cleavage surfaces of sodium-chloride crystals and potassium-chloride crystals, *Gahm* [20] shows that DIC microscopy can be used to advantage for quantitative investigations. Slipbands that cannot be recognized under bright-field illumination stand out with extraordinary clarity in the DIC image.

The superiority of the Nomarski DIC method over phase contrast is clearly evident from the replica of a calcite cleavage surface [18, 19].

Padawer [39] compares sodium-chloride crystals in bright field, phase contrast and differential interference contrast. In bright field practically only the contours of the crystals will become visible, even if the illuminating aperture is reduced, and in the phase-contrast image, large parts of the object will be veiled by halation.

For the optical staining and examination of the surface of germanium and silicon oxide, *Françon* [17] takes recourse to reflected-light differential interference contrast. However, ammonium-alum crystals can be reproduced with a wealth of detail by optical staining even when transmitted light is used.

Mineralogy

Gahm [20] has successfully used Nomarski DIC microscopy to make microhardness impressions in various minerals (e. g. covellite, boulangerite) visible. Cracks, scabbiness and bulges around a microhardness indentation in a periclase cleavage surface can be made optimally visible by color contrast. *Von Gehlen* and *Piller* [21] have shown that Nomarski DIC microscopy is an ideal means for examining polished specimens or ore minerals for hardness differences. This method has proved to be superior to the conventional "Schneiderhöhn line". Moreover, the Nomarski method is a valuable aid in testing the quality of polished surfaces whose reflectivity is to be measured by microphotometry. Finally, with sub-stage illumination the Nomarski method offers many advantages for assessing the morphographic properties of fine-grain minerals and identifying clay minerals, as *Correns* and *Piller* [11] have shown.

Semiconductor technology

In the introduction to his paper, *Le Méhauté* [24] describes the fundamentals and characteristics of the Nomarski differential interference-contrast method and continues by giving a summary of its advantages over bright-field and phase-contrast observation, particularly for metallographic uses. In the semiconductor field the DIC technique may be used to advantage for observing structural changes such as phase transitions, the formation of new phases, recrystallization processes, etc. *Le Méhauté* shows that the DIC image of a transistor reveals far more detail than would a bright-field image.

Besides a comprehensive and easily understandable introduction to Nomarski DIC microscopy, *Françon* [17] publishes a number of photomicrographs of a germanium surface, a microcircuit and a cadmium-telluride film on silicon dioxide.

Owing to its high resolution, the Nomarski method is well suited for the examination of silicon monocrystals, as has been shown by *Vieweg-Gutberlet* [45]. If the specimens are properly etched, inhomogeneities in the doping concentrations such as striations, the microstructures of striations, stacking faults in concentrations etc. can be made visible. Fig. 4 shows a wafer in bright field (left) and in Nomarski differential interference contrast (right).

Fig. 5 shows a cross-section of an integrated circuit that failed due to overload, once in bright field (a) and once in DIC (b). Differential interference contrast above all reproduces the conductors with greater detail.

Glass technology

Minute details in a glass surface are reproduced with high contrast in the DIC image. As is proved by *Gabler* and *Herzog* [18, 19], pronounced differences of refractive index between object and mounting medium which, in phase contrast, result in extremely disturbing haloes, do not have the same unfavorable effect in the DIC image.

When examining oriented linear phase structures, their orientation relative to the splitting direction of the Nomarski prisms must be taken into account [28]. The phase-contrast technique has no inherent azimuth effect. However, it has the disadvantage of greater depth of field so that phase objects lying outside the focal plane will appear in the image, for example, as disturbing diffraction fringes.

Plastics

Like clear transparent glass, clear transparent plastics are ideal phase objects. According to *Gahm* [20], microhardness impressions of extremely minute irregularities that are invisible in bright field can be emphasized by suitable setting of the background, above all in color contrast.

A comparison between the optical staining of spherical plastic parts in dark field and differential interference contrast is made by *Padawer* [39]. This comparison proves the advantages of the DIC method by which even very small particles are clearly reproduced beside larger objects. With the aid of polystyrene spheres with a mean diameter of 1.3 μm, mounted in glycerin, *Padawer* demonstrates the dependence of the DIC image on the illuminating aperture. The optimum illuminating aperture depends both on the object and the illuminating aperture. In the special case under discussion, a setting of 75 % of the maximum aperture has proved particularly favorable. In addition, with the aid of two photomicrographs, the author explains the effect of defocusing on the DIC image. A number of micrographs of plastic spheres shows that diffusion processes within the particles, involving a variation of optical thickness, can be clearly reproduced by differential interference contrast.

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