Chapter 11
Differential Interference Contrast Microscopy

Many microscopic specimens are colorless, nearly transparent, and relatively thick, such as tissue sections. This chapter presents a method for producing contrast in this type of specimen without the artifacts associated with phase contrast. The resulting image is one that has a very "topographic" appearance. It looks as though you are looking down on the specimen while it is being lit strongly from one side. Figure 11.0 is a DIC image of cheek epithelial cells.

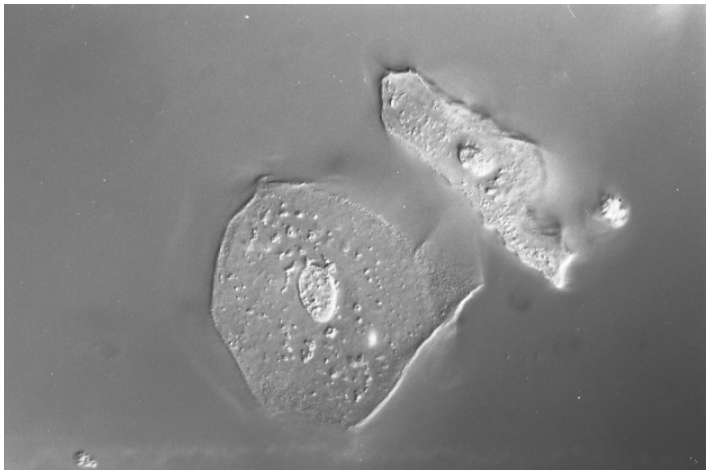


Figure 11.0 DIC of Cheek Epithelial Cells

Differential Interference Contrast (DIC) is optically a rather complicated method requiring several special optical components. These are described and their action in producing this beautiful image is discussed in the following sections.

Types of Specimens Suitable for DIC

Those specimens that would be suitable for phase contrast microscopy are also suitable for DIC. Furthermore, DIC produces clearer images of relatively thick specimens than does phase contrast. Cytological, histological, microbiological, and cell culture specimens are of this type as are chromosome spreads. DIC is especially useful in fluorescence and confocal microscopy to indicate the morphological aspect of fluorescent regions. DIC reveals some ultrastructural features of cells such as microtubules and cytoplasmic granules. When coupled with enhanced video techniques, DIC enables the visualization of actin structures that are actually below the resolution of the microscope. Finally, the use of DIC on normal, stained or unstained histological sections can provide additional useful information.

History of DIC

We owe the current method of DIC to the work of Georges Nomarski (figure 11.1) who developed it in the 1960's. It is based on an older method of interferometery microscopy in which a beam of light is split in two sending one beam through the specimen and the other through the same optical length path but without the specimen. The beams were then recombined to produce an interference pattern. Nomarski was able to produce a modified Wollaston prism that he could place directly in the back focal plane of high magnification, high NA objectives. He altered the older method by using two beams of light that had a separation distance smaller than the resolution of the objective. The recombination of the two beams produced an enhanced image of the specimen rather than an interference pattern.

How DIC Works in Summary

Figure 11.1
Georges Nomarski

Before tackling the specifics of Nomarsky DIC, a quick summary of the technique will help organize the sections that follow.

In DIC, the illumination starts as a plane polarized beam of light. This is divided into two beams that have different polarization angles and that are separated horizontally by a distance roughly equal to the resolution of the objective lens. As these two beams pass through a specimen, changes in refractive index due to the fine structure of the specimen affect one beam more than the other as shown in figure 11.5. After passing through the specimen and the objective lens, the horizontal separation between the two beams is removed. In the final step, the two beams are converted to the same polarization angle. At this point, in the microscope's intermediate image plane, the two beams can interfere with one another to produce amplitude contrast. This contrast reflects the optical path difference between the two beams that was introduced by the specimen. Because the initial separation or shear between the beams is so small, the technique tends to be very sensitive to changes in the curvature of specimen structures and to refractive index gradients. The differential in DIC is the difference in optical path length of the two beams.

Properties of Light Involved in DIC

In addition to the properties of light discussed with phase contrast microscopy, DIC also employs the polarization of light from the outset. Two plane polarizers are involved, one before the condenser and one after the objective. Birefringent prisms act on the plane polarized light first to split the beam in two, and later to recombine the two beams.

Polarization Angle

In Nomarski DIC, unpolarized light from the lamp is first plane polarized. Usually a dichroic material such as Polaroid does this. Dichroic materials absorb light. The amount absorbed depends on the light's vibration axis (i.e., polarization angle) relative to the major transmission and absorption axes of the polarizer. Light vibrating in the Polaroid's major absorption axis will be nearly all absorbed whereas light vibrating at 90 degrees to this axis will be nearly all transmitted. Light vibrating at other angles will be partially absorbed and partially transmitted. All transmitted light will have the same vibration orientation as the polarizer's major transmission axis . An important property of polarized light as used in DIC is that interference can not occur between partially coherent beams of polarized light that have different polarization angles. Figure 11.2 illustrates the action of a polarizer. In this illustration, think of the light as coming toward you out of the page. The polarizer's major transmission axis (vibration axis) is shown vertical; its major absorption axis is horizontal. Light can enter at any rotational angle. In the left hand panels, the vertical and horizontal lines are vectors representing the amount of light that is transmitted and absorbed for light rays entering at different angles – the middle line. The right hand panels represent the relative amount of light you would see while looking through the polarizer.

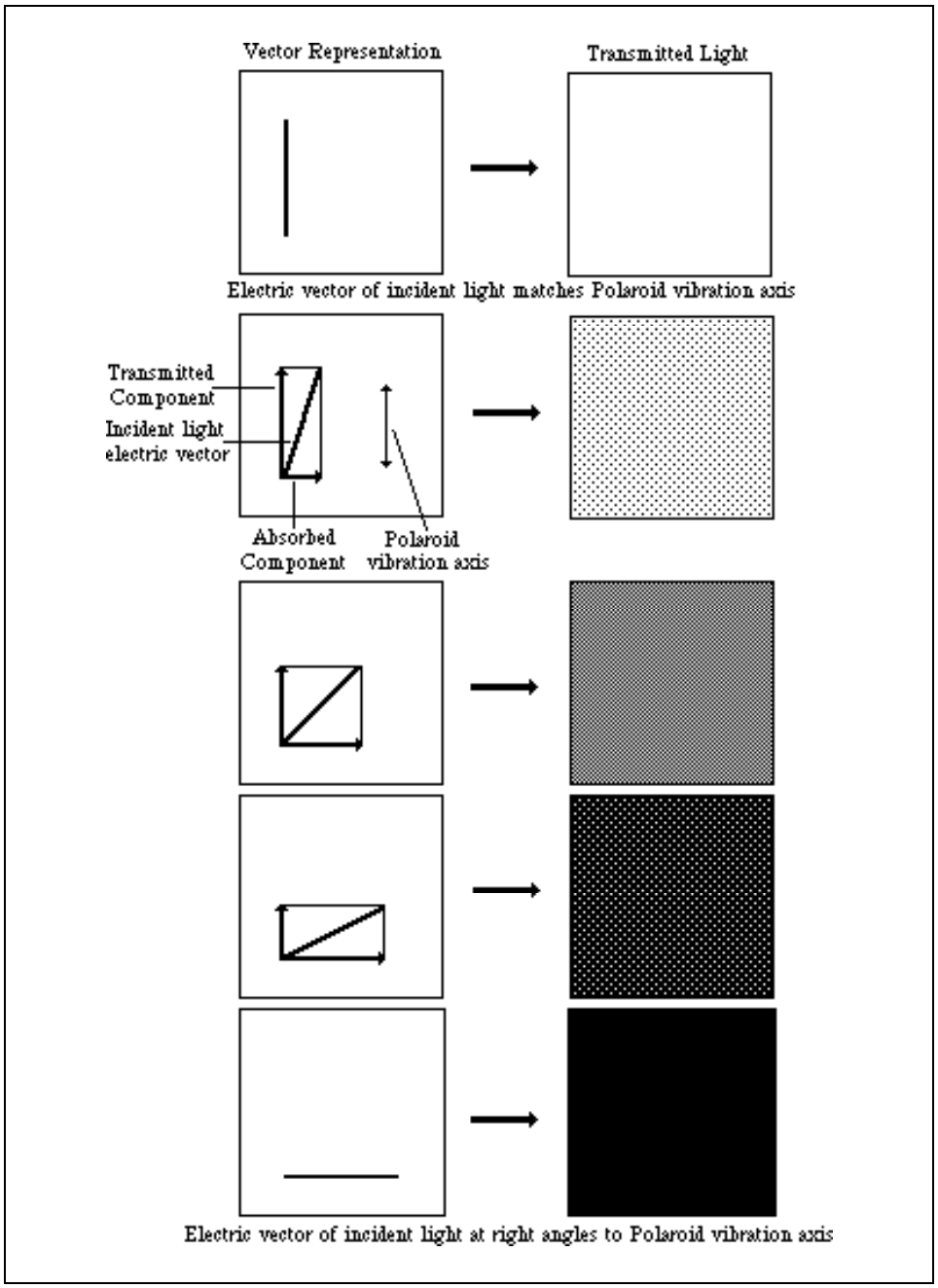


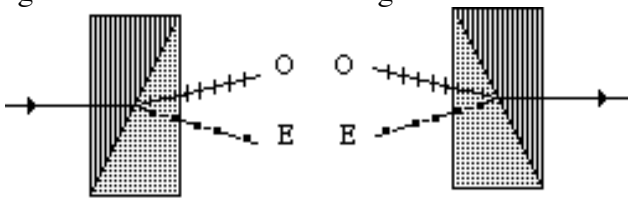
Figure 11.2 - Action of a Polarizer.

Beam Splitting by a Birefringent Prism

Birefringent materials have the capacity to split a beam of light into two beams polarized at right angles to one another. One of these rays is the ordinary ray or O ray. The other ray is the extraordinary ray or E ray. One of these rays travels more slowly through the birefringent material than the other and therefore the two rays emerge with a slight difference in phase. As shown in figure 11.3, a prism can be constructed using birefringent materials in which the two rays emerge traveling in slightly different directions (the divergence is greatly exaggerated in the drawing for clarity). This difference is referred to as shear between the beams. Nomarski used a modified

Wollaston prism to achieve this effect. The emergent beams from the Wollaston prism cannot interfere with one another because their polarization angles differ, they are slightly sheared because they are traveling in slightly different directions, and they are slightly out of phase.

Figure 11.3 Action of Birefringent Prisms



Beam Straightening and Beam Converging

The condenser acts to straighten, that is to make parallel, the diverging beams after the first Wollaston and the objective lens serves to converge the beams before the second Wollaston.

Beam Combining by a Birefringent Prism

If an O ray and E ray pass into a Wollaston prism that has the opposite arrangement of the one that made them, what emerges form the second Wollaston will be two beams of light that are polarized at right angles to each other but which are not sheared. The phase relationship of the two beams can be adjusted by shifting the position of the final prism across the two beams (Figure. 11.3). For example, moving the right prism down would slow the O ray more than the E ray.

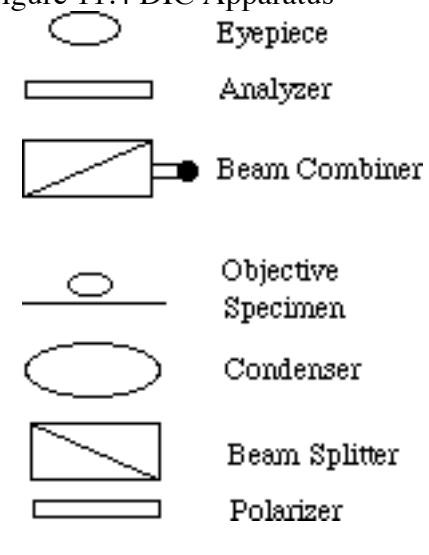
Resolution of two Polarized Beams by an Analyzer

If two beams of light, polarized at different angles, enter a polarizer, light will emerge at a single polarization angle. Interference of the two original beams is now possible. A polarizer used in this capacity is called an analyzer.

Apparatus for Nomarski type DIC

Figure 11.4 illustrates the apparatus commonly used for Nomarski DIC. Starting at the bottom and working up, the apparatus is as follows:

Figure 11.4 DIC Apparatus



1) Polarizer – This produces a single beam of plane polarized light from the lamp.

2) Modified Wollaston prism – This produces two beams of light, polarized at right angles to one another, slightly out of phase with one another, and having a very small amount of shear. The amount of shear is on the order of the resolution of the objective lens.

3) Condenser – The condenser focuses the two

slightly sheared beams on the specimen plane. DIC requires that the microscope be aligned for Köhler illumination.

4) Specimen – The specimen causes a change in optical path difference between the two beams. This change is sensitive to very closely spaced variations in the specimen's refractive index and/or thickness since the beams themselves are very closely spaced.

5) Strain free plan achromatic objectives – A DIC objective lens must not affect the polarization of light. This can happen if the glass elements of the lens have been subjected to stresses that cause slight variations in the refractive index. It is difficult to manufacture an apochromatic lens that is strain free because of the large number of lens elements. Treat your strain free lenses very gently. One jolt can ruin them.

6) Second modified Wollaston prism – A second prism is placed above the objective lense. The location of the interference fringes of this Wollaston prism must coincide with the back focal plane of the objective. The back focal plane of middle and high power objectives is inside the objective. The Wollaston prism therefore has its interference fringes located outside the prism. Specific prisms are matched to specific lenses. It is therefore unlikely that any objective lens you might have will function in a DIC system. Rather you should buy prisms and objectives that are made for DIC. Manufacturers usually indicate DIC on the barrel of these objectives. The upper modified Wollaston prism(s) will be movable in a lateral direction. Moving this prism horizontally affects the optical path difference between the two beams and therefore the amplitude contrast in the final image. It emphasizes optical path differences introduced by the specimen.

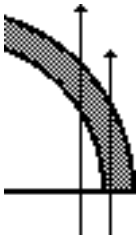
7) Analyzer – This is a second polarizer placed after the second modified Wollaston prism. This polarizer has its major transmission axis at right angles to the lower polarizer.

8) Eyepiece – Interference of the two modified beams occurs in the intermediate image plane forming a real image that is further magnified by the eyepiece.

Effect of the DIC Apparatus

The image that results from all this apparatus is one that is very sensitive to refractive index gradients. Figure 11.5 illustrates the affect that a cell membrane would have on the two slightly sheared beams. The right-hand beam is in the cell membrane longer than the left-hand beam. The cell membrane has a higher refractive index than the cytoplasm. The right-hand beam slows down relative to the left-hand beam. The longer the right-hand beam remains in the cell membrane, the greater its optical path difference (opd) will be from the left hand beam. The second Wollaston prism removes the separation between the two beams. The analyzer recombines these beams at the same polarization angle. The beams now interfere with one another and the membrane is clearly visible. If the beams in figure 11.5 are shifted to the left, the opd would not be as

Figure 11.5 OPD



great and the image would be different. It is often said that DIC is sensitive to wedges of refractive index difference.

Set Up of the DIC Apparatus

Correct placement and orientation of the various parts of a DIC system are crucial. The polarizer and analyzer must have their major transmission axes at right angles to one another and the modified Wollaston prisms must be arranged optically opposite one another and oriented at 45 degrees to the polarizers. Figure 11.4 illustrates the optical arrangement of these components.

Use the following steps in setting up a DIC system:

1) Using a 40 X objective, align the microscope for bright field Köhler illumination.

2) Move the specimen to obtain an empty field of view on the slide.

3) Fully open the aperture iris diaphragm.

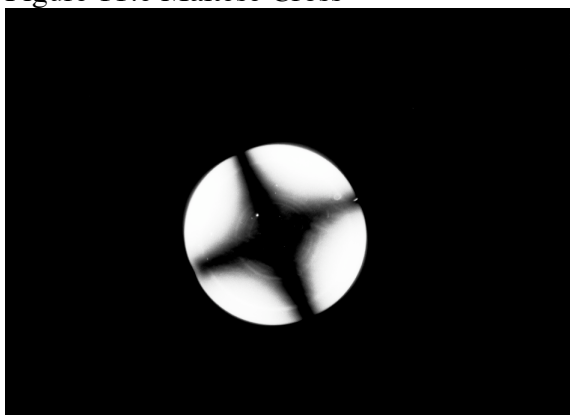
4) Insert the polarizer and the analyzer – insure that they are right side up. Usually this is indicated on the mount. If the polarizer and/or analyzer are rotateable, make sure the polarizer is set to the 0 or 90 degree position and that the analyzer is set to the opposite position. Usually the polarizer's major transmission axis is set right to left across the microscope and the analyzer's is set front to back.

5) Make sure neither Wollaston prism is in position. That is, set the condenser for bright field operation and remove or retract any Wollaston prism on the back side of the objective lens.

6) Focus on the objective's back focal plane. Use a phase telescope, or a Bertrand lens to do this.

7) You should see a dark cross in an otherwise bright field (figure 11.6). If you do not, loosen the analyzer housing and rotate it slightly until the cross appears. Then, lock the housing in place. (Your microscope may use a different method.) This insures that the polarizer and analyzer are exactly at right angles to one another.

Figure 11.6 Maltese Cross



8) Return to normal viewing and insert the Wollaston prisms. On some systems, the condenser contains only a single prism and each objective has a prism placed just above it. In this case it is a good idea to see that the prisms over the objectives are the correct

ones. These prisms will be marked with the magnification of the objective for which they are made. On other systems, the condenser contains the objective-matched prisms. In this case the condenser turret positions will be labeled with some indication of matching objectives.

9) Insert your specimen and adjust the illumination. Move the adjustable Wollaston prism to achieve the desired DIC effect.

Comparison of DIC with Phase Contrast Microscopy

The phase contrast artifacts of phase halo and shading off are absent in the DIC image. The image has a very realistic three-dimensional appearance. This appearance is not an entirely true representation of the specimen. You only have to adjust the movable Wollaston prism and observe the change in appearance of the specimen to prove this. It is therefore a good idea to compare the appearance of the specimen by phase contrast and DIC to achieve a better understanding of specimen structure.

Because the DIC technique is designed to work at the limits of resolution of the objective lens, higher image resolution can be achieved than with phase contrast. A significant advantage of DIC is its ability to produce very clean optical sections compared to phase contrast – that is, its resolution in the z-axis is much better than phase.

Although phase contrast requires additional optical equipment, the DIC apparatus is more complex and generally more expensive.

Problems with DIC

DIC is a relatively expensive technique. The microscope stand must be able to accept the DIC apparatus. Special, strain free, objective lenses are recommended, and there is the expense of the associated filtering systems.

Specimens that alter polarized light, such as birefringent specimens and plastic culture dishes, are not suitable for DIC. Phase contrast is still the best method for examining cells in plastic culture dishes. A method called Hoffman Modulation Contrast can be used to produce an image similar to DIC for specimens in plastic dishes, but this method is also expensive.

Exercises

1) Set your microscope up for DIC. Note where the following components are located: polarizer, first Wollaston prism, second Wollaston prism, and analyzer. Make a rough sketch of their positions.

2) Check the alignment of your DIC system. What method is available for adjusting the system for crossed polars?

3) Observe cheek cells using DIC. Describe how the image compares to a phase image?

4) Move the adjustable Wollaston prism from one extreme to the other while observing cheek cells. What happens to the image?

5) Remove one of the DIC optical components while looking at cheek cells. What is the effect on the image?