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Technical Note

Using SGNPs in Darkfield Microscopy To Enhance Contrast: An Easy and Inexpensive Method

TN804

INTRODUCTION[1]

Light microscopy is an important investigative tool for biology that is used regularly in high schools and colleges. The structure of many biological specimens are of low contrast that cannot be revealed by the brightfield compound microscopes which are provided in many classrooms. Microscopes that improve the contrast of these specimens through special optics are prohibitively expensive for most teaching budgets. This article describes a simple, inexpensive modification that changes a brightfield microscope into a darkfield microscope allowing low contrast samples to be examined. This article explains the basic theory of darkfield microscopy. Photographs comparing brightfield and darkfield application are presented. This technique allows expanded use of the brightfield microscope at all levels of teaching with very little manipulation or expense.

Theory of darkfield microscopy

Microscopes are used to magnify objects. Through magnification, an image is made to appear larger than the original object. The magnification of an object can be calculated roughly by multiplying the magnification of the objective lens times the magnification of the ocular lens. Objects are magnified to be able to see small details. There is no limit to the magnification that can be achieved; however, there is a magnification beyond which detail does not become clearer. The result is called empty magnification when objects are made bigger but their details do not become clearer. Therefore, not only magnification but resolution is important to the quality of the information in an image.

The resolving power of the microscope is defined as the ability to distinguish two points apart from each other. The resolution of a microscope is dependent on a number of factors in its construction. There is also an inherent theoretical limit to resolution imposed by the wavelength of visible light (400-600nm). The theoretical limit of resolution (the smallest distance able to be seen between two points) is calculated as:

$$\text{Resolution} = 0.61\,\lambda/\text{N.A.}$$

where λ represents the wavelength of light used and N.A.is the numerical aperture. The student-grade microscopes generally have much lower resolution than the theoretical limit because of lower quality lenses and illumination systems.

[1] http://www.wsu.edu/~omoto/papers/darkfield.html

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Standard brightfield microscopy relies upon light from the lamp source being gathered by the substage condenser and shaped into a cone whose apex is focused at the plane of the specimen. Specimens are seen because of their ability to change the speed and the path of the light passing through them. This ability is dependent upon the refractive index and the opacity of the specimen. To see a specimen in a brightfield microscope, the light rays passing through it must be changed sufficiently to be able to interfere with each other which produces contrast (differences in light intensities) and, thereby, build an image. If the specimen has a refractive index too similar to the surrounding medium between the microscope stage and the objective lens, it will not be seen. To visualize biological materials well, the materials must have this inherent contrast caused by the proper refractive indices or be artificially stained. These limitations require instructors to find naturally high contrast materials or to enhance contrast by staining them which often requires killing them. Adequately visualizing transparent living materials or thin unstained specimens is not possible with a brightfield microscope.

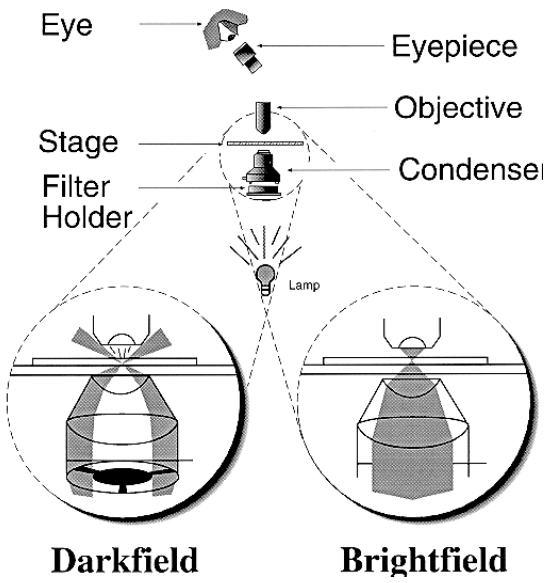

Figure 1.
This diagram compares the essential components of brightfield and darkfield microscopy. The difference in illumination (shown by stippling) of the sample between brightfield and darkfield is emphasized in the diagram. Darkfield utilizes a darkfield "stop" illustrated by the "spider stop" placed below the condenser. This stop blocks the center of the beam of light to produce a hollow cone of light. This light does not directly enter the objective lens. In contrast, a solid cone of light illuminates and enters the objective lens in brightfield. Only light that is scattered by the sample (depicted by the lines in the diagram) and enters the objective lens is seen as an image in darkfield.

Darkfield microscopy relies on a different illumination system. Rather than illuminating the sample with a filled cone of light, the condenser is designed to form a hollow cone of light. The light at the apex of the cone is focused at the plane of the specimen; as this light moves past the specimen plane it spreads again into a hollow cone. The objective lens sits in the dark hollow of this cone; although the light travels around and past the objective lens, no rays enter it (Figure 1). The entire field appears dark when there is no sample on the microscope stage; thus the name darkfield microscopy. When a sample is on the stage, the light at the apex of the cone strikes it. The image is made only by those rays scattered by the sample and captured in the objective lens (note the rays scattered by

the specimen in Figure 1). The image appears bright against the dark background. This situation can be compared to the glittery appearance of dust particles in a dark room illuminated by strong shafts of light coming in through a side window. The dust particles are very small, but are easily seen when they scatter the light rays. This is the working principle of darkfield microscopy and explains how the image of low contrast material is created: an object will be seen against a dark background if it scatters light which is captured with the proper device such as an objective lens.

The highest quality darkfield microscopes are equipped with specialized costly condensers constructed only for darkfield application. This darkfield effect can be achieved in a brightfield microscope, however, by the addition of a simple "stop". The stop is a piece of opaque material placed below the substage condenser; it blocks out the center of the beam of light coming from the base of the microscope and forms the hollow cone of light needed for darkfield illumination.

Procedures

One example of a darkfield stop is shown in Figure 1; this is a "spider" stop available for an Olympus CH series brightfield student-grade microscope. The Olympus system has a color filter holder below the substage condenser which can be removed; the color filter is replaced with the darkfield stop and the holder is placed back into position. Other manufacturers have slightly different designs. Some manufacturers include this simple stop with the purchase of the microscope.

LIST OF MICROSCOPE MANUFACTURERS

1. Carl Zeiss USA
   Microscopy Division
   1-800-233-2343
   (ask for name and number of local representative)

2. Leica Microsystems Incorporated
   111 Deer Lake Road
   Deerfield, IL 60015
   1-800-248-0123
   (includes American Optical, Bausch and Lomb, Leitz, Reichert, and Wild products)

3. Meiji Techno America
   2186 Bering Drive

San Jose, CA 95131-2013
1-408-428-9654

4. Nikon, Incorporated
   Instrument Group
   1300 Walt Whitman Road
   Melville, NY 11747-3064
   1-516-547-8500

5. Olympus America Incorporated
   Precision Instruments Division
   4 Nevada Drive
   Lake Success, NY 11402-1179
   1-800-455-8236

or contact known area representatives of these companies.

If a manufactured darkfield stop is not available for your microscopes, there are some alternatives. If there is a filter holder below the condenser, a dark field stop from another company may fit or be made to fit. A coin or a circle of other opaque material can be mounted in the center of a clear disk, e.g., glass, and inserted. A stop can also be fashioned by punching out a circle of black construction paper; the circle is attached to the bottom of the condenser with double-stick tape. This alternative can be a bit tricky because the material needs to be placed in the center of the condenser and the condenser needs to be cleaned when the tape is removed. Technically, to properly block the beam, the stop should vary in diameter from 8mm to 20mm depending on the magnification and numerical aperture of the objective lens.

Darkfield microscopy reduces the amount of light entering the lens system of a microscope in two ways. First, the stop blocks the center of the beam of light that would otherwise fill the objective lens. Second, only the light which is scattered by the specimen and enters the objective lens is seen. Therefore, the best viewing result requires increasing the light intensity as much as possible: by setting the light intensity adjustment at maximum, by opening the field diaphragm, by opening the condenser aperture, and by removing any color or other filters. The correct microscope slides also should be used; they should be 1mm thick.

The illumination needs to be aligned and adjusted to achieve the best image. Before making the darkfield modification, align the light beam in the center of the field of view according to the manufacturer¹s instructions. To facilitate focusing the substage condenser and the objective lens, use a slide filled with samples that are easy to find;

instructions for making a cheek cell slide follow. Focus the sample slide at low magnification (10X) in the brightfield mode. Insert the darkfield stop without changing the focus. Make sure that the maximum amount of light is available. Rack up the condenser to its highest position with the condenser focus knob. Look at the sample and slowly lower the condenser until the sample is visible against a dark background and in sharpest contrast. Finally, adjust the view of the image with the fine focus knob.

Limitation of darkfield microscopy

The advantage of darkfield microscopy also becomes its disadvantage: not only the specimen, but dust and other particles scatter the light and are easily observed. For example, not only the cheek cells but the bacteria in saliva are evident in Figure 2D. More care in sample preparation needs to be exercised in darkfield application. Glass slides need to be thoroughly cleaned of extraneous dust and dirt. It may be necessary to filter sample media (agar, water, saline) to exclude confusing contaminants. Sample materials need to be spread thinly; too much material on the slide creates many overlapping layers and edges making it difficult to interpret structures.

To create the image, this technique relies on scattered light from specimens. Color is lacking or minimal; this can be disappointing to the viewer. The actual size of specimens is also impacted; the width of objects becomes exaggerated.

Examples of darkfield images

Figure 2 compares the images of living Chlamydomonas, a biflagellate algae in brightfield and in darkfield modes. Although the cells are seen with brightfield, the flagella are not discernible (Fig. 2B). Some contrast can be achieved by closing down the condenser aperture diaphragm which then allows the flagella to be seen (Fig. 2A). However, closing down the condenser aperture decreases the numerical aperture of objective lens effectively reducing the resolution. Darkfield allows flagella and details inside the to be seen distinctly (Fig. 2C). The use of darkfield microscopy thus achieves both high contrast and high resolution.

Cheek cells make a quick study. Buccal cells are obtained by gently scraping the inside of the mouth with a toothpick and thinly spreading the cells on a slide; a cover slip is placed over the preparation which is not allowed to dry. These cells have no inherent contrast and are difficult to see with a brightfield. Dramatic contrast is achieved in a darkfield microscope; the nucleus and other intracellular inclusions as well as bacteria in the surrounding medium can be easily located and identified (Fig. 2D).

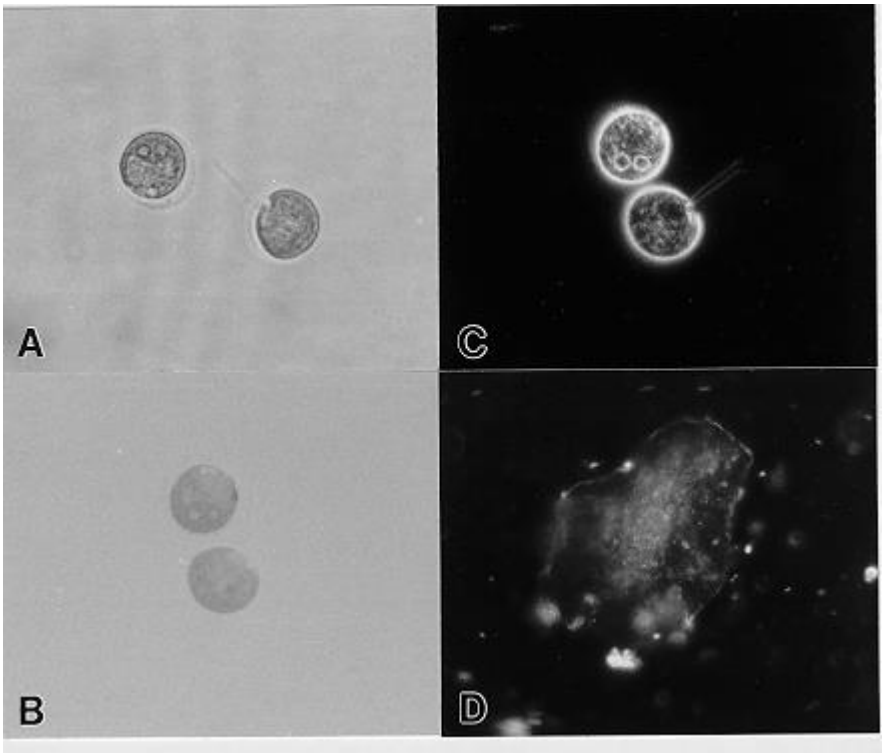

Figure 2.

Photomicrographs of Chlamydomonas are shown in Figure 2A-C and a buccal cell in Figure 2D. The difference in refractive indices of the media with the cell wall and other components of the algae are visualized in brightfield (Fig. 2A & B). Closing the condenser aperture diaphragm increases contrast allowing the flagella to be barely visible (Fig. 2A). Darkfield clearly shows many intracellular details as well as the flagella (Fig. 2C). Because these are unmounted living cells, they have moved a bit between the different exposures. Buccal cells are so similar in refractive index to the medium that photos of them could not be obtained in brightfield. Figure 2D shows buccal cells in darkfield; nucleus and other intracellular structures are clearly visible. Samples were photographed using CH2 student-grade Olympus microscope with 40X objective and 3.3X photo eyepiece on Kodak TMAX 400 film.