Using Darkfield Microscopy To Enhance Contrast

An Easy & Inexpensive Method

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Light microscopy is an important investigative tool for biology that is used regularly in high schools and colleges. However, many biological specimens are of low contrast and cannot easily be visualized by brightfield compound microscopes which are provided in many classrooms. Microscopes that improve the contrast of these specimens through special optics are usually 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 also explains the basic theory of darkfield microscopy. Photographs of common specimens comparing brightfield and darkfield application are presented. This technique allows the 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. 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, both resolution and magnification are important to the quality of the information in an image.

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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 the resolution imposed by the wavelength of visible light (400 to 600 nm). The theoretical limit of resolution (the smallest distance able to be seen between two points) is calculated as:

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

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

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 (Figure 1). As light travels through the specimen, its speed and path changes. These changes are dependent upon the refractive index and the opacity of the specimen. For a specimen to be seen in a brightfield microscope, the light rays passing through it must be changed sufficiently to be able to interfere with each other. This interference produces contrast (differences in light intensities) and, thereby, builds an image. If the specimen has a refractive index too similar to the surrounding medium, as most biological specimens do, it will not be seen. To be visualized well, biological materials must have inherent contrast created 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 the specimen. Adequately visualizing transparent living materials or very thin unstained specimens is not possible with a brightfield microscope.

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 object 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

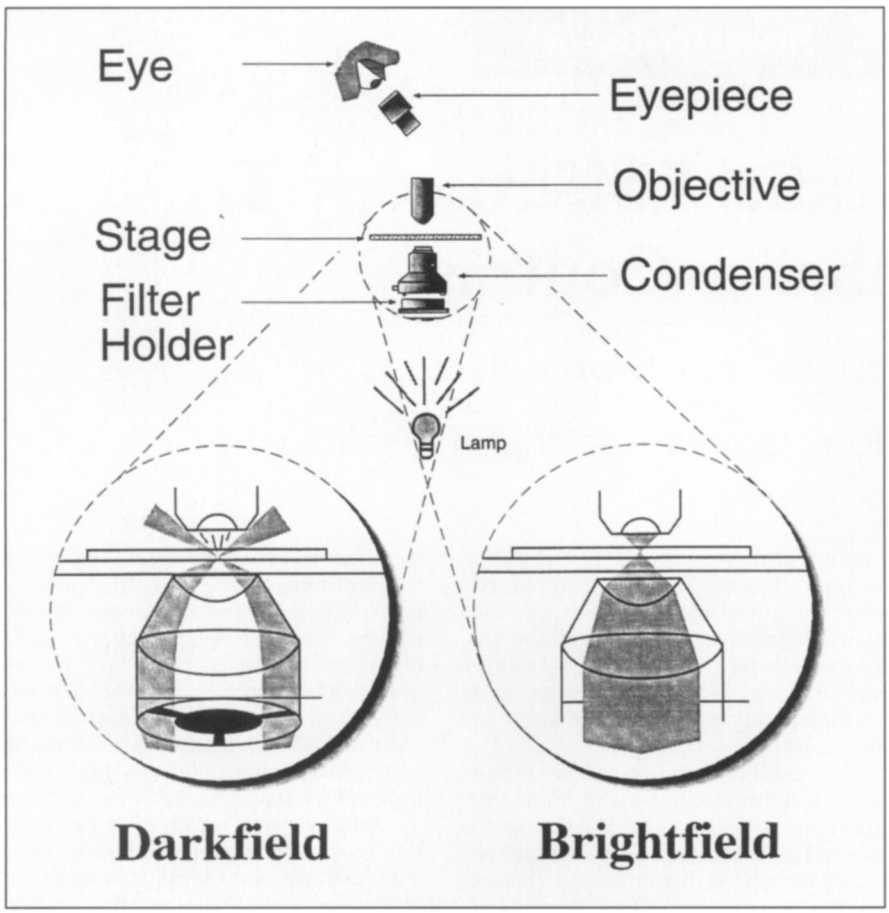

Figure 1. This diagram compares the essential components of brightfield and darkfield microscopy. The difference in illumination (shown by stippling) of the sample between brightfield and darkfield is emphasized in the diagram. Darkfield utilizes a darkfield "stop" illustrated by the "spider stop" placed 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. 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. In contrast, a solid cone of light illuminates and enters the objective lens in brightfield.

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 lamp and forms the hollow cone of light needed for darkfield illumination.

Procedures & Applications

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 filter holder below the substage condenser that can be removed; the filter is replaced with the darkfield stop and the holder is placed back into position. Other manufacturers have slightly different designs. Most manufacturers will include this simple stop with the purchase of the microscope. In the case of the Olympus CH series, a spider stop purchased separately costs approximately $20. [A list of manufacturers is provided in the Appendix.]

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 darkfield stop from another company may fit or be made to fit, or a circle of other opaque material can be mounted in the center of a clear disk, e.g. glass, and inserted. If a filter holder is not available, a stop can be fashioned by punching out a circle of black construction paper; the circle is then directly 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 8 mm to 20 mm, depending on the magnification and numerical aperture of the objective lens. A stop with a diameter of 18 mm should produce a darkfield effect satisfactory for most classroom use.

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 that is scattered by the specimen and enters the objective lens is seen. Therefore, the best 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 diaphragm, and by removing any color or other filters. The proper microscope slides also should be used: they should be 1 mm ± 0.1 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 manufacturer's 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.

The advantage of darkfield microscopy also becomes its disadvantage: not only the specimen, but also dust and other particles scatter the light and are easily observed. For example, bacteria surrounding the cheek cells

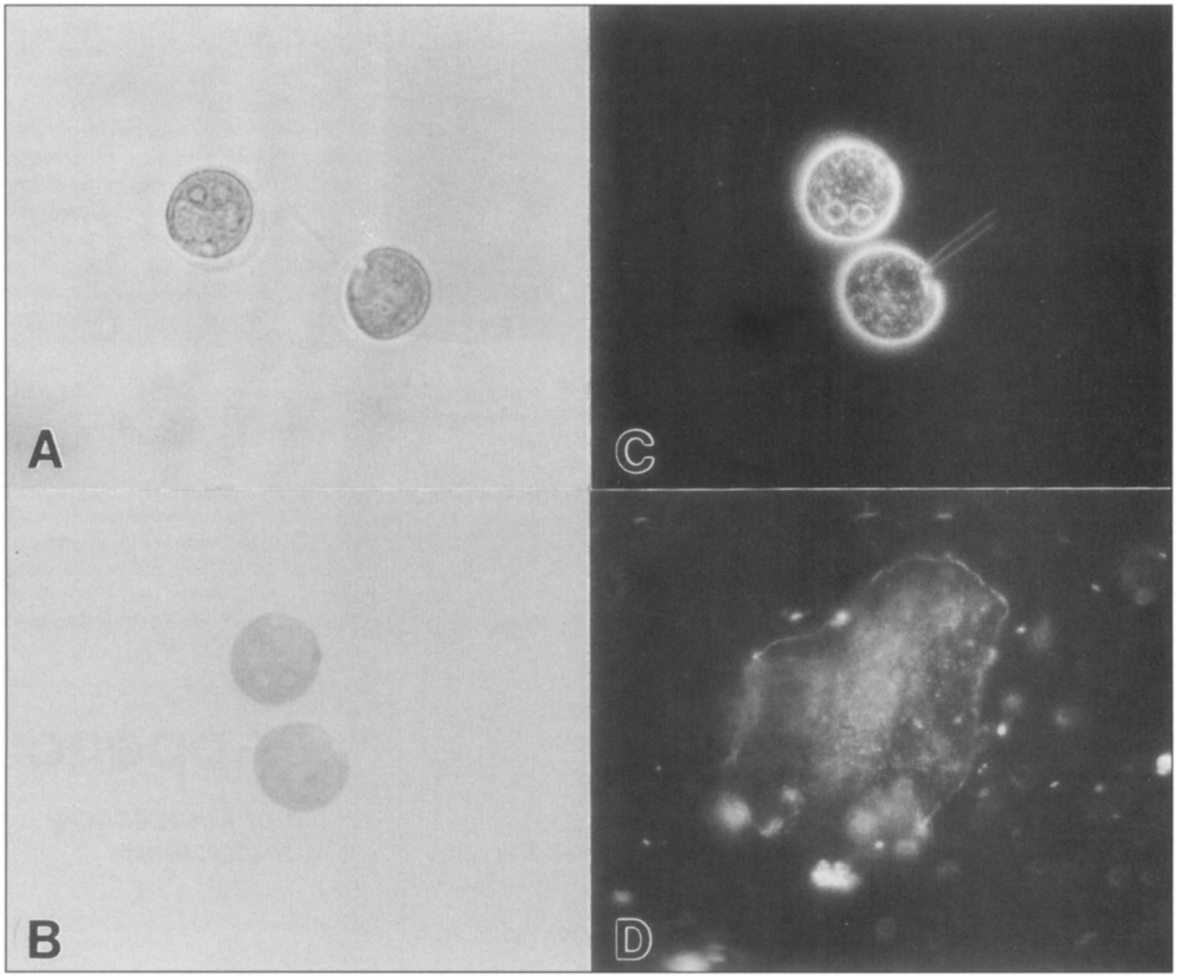

Figure 2. Photomicrographs of *Chlamydomonas* are shown in Figure 2A-C and a buccal cell in Figure 2D. The difference in refractive indices of the media with the cell wall and other components of the algae is visualized in brightfield (Figure 2A & B). Closing the condenser aperture diaphragm increases contrast allowing the flagella to be barely visible (Figure 2A). Darkfield clearly shows many intracellular details as well as the flagella (Figure 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.

in the saliva are quite evident in Figure 2D. Therefore, more care in sample preparation needs to be exercised in darkfield application. Glass slides and cover slips must be cleaned of extraneous dust and dirt. Sample materials need to be spread thinly; too much material on the slide creates too many overlapping layers and edges, making it difficult to interpret structures.

There are particular characteristics exhibited by darkfield images. Since this technique relies on scattered light, color is lacking or minimal; this can be disappointing to the viewer. In addition, the actual dimensions of a specimen are distorted because its width is exaggerated.

Darkfield microscopy provides unique views of cells. For example, a brightfield microscope provides a view of *Chlamydomonas* cells, but not their flagella (Figure 2B). The flagella can be visualized when contrast is increased by closing down the condenser aperture diaphragm (Figure 2A). However, closing down the condenser aperture diaphragm decreases the numerical aperture, effectively reducing the resolution. A darkfield microscope, however, clearly shows the flagella and details inside the cells (Figure 2C). Other cells easily observed are cheek cells. These cells are obtained by gently scraping the inside of the mouth with a toothpick and thinly spreading them on a slide; a cover slip is placed over the wet preparation. These cells have no inherent contrast and are difficult to see in brightfield mode. Dramatic contrast is achieved in the darkfield mode; the nucleus and other intracellular inclusions as well as bacteria in the surrounding medium can be clearly seen (Figure 2D). Using darkfield microscopy achieves high contrast without sacrificing resolution.

The simple method described here to achieve the darkfield effect greatly expands the application of brightfield microscopes already existing in a classroom. This method is advantageous for viewing transparent and semitransparent specimens, such as diatoms, foraminifera, hydra, and insects and insect parts. Very small specimens such as bacteria, spermatozoa and protozoa are difficult to visualize in brightfield, or with phase contrast. Cilia of protozoa and sperm tails are really only clearly visible using darkfield. Most of these specimens are best viewed in an aqueous medium.

Finally, an unquestionable advantage is that students can observe living organisms and processes without staining. For example, it is possible to observe the regeneration of cilia on algae without staining. Thus using darkfield microscopy, classroom experiments can be developed that allow students to observe processes in real time.

Summary

A brightfield microscope can be modified very inexpensively to have darkfield capability. There are three particular benefits of darkfield microscopy. One, structures of transparent and semi-transparent specimens can be visualized without staining. Two, very small objects such as flagella can be visualized. Last, but not least, students can observe processes in living cells.

Appendix

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.