

## Chapter 9

# Dark Field Microscopy

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In the 1840's, the ultimate test objects for light microscopes were diatoms, in particular the species then known as Navicula Spencerii. This diatom was named after Charles Spencer, the New York lens maker whose lenses could resolve this diatom's striae. It was found that the use of oblique light was required to resolve the striae. **Oblique illumination** was the first step toward dark field microscopy. In dark field, light seems to radiate from the specimen while all the rest of the field is black. Resolution is as good as that in bright field while contrast is enhanced.

Syphilis is what made dark field popular. Schaudinn discovered Treponema pallidum – the syphilis spirochete – in 1905 using dark field microscopy. This promoted the use of dark field by the medical profession and subsequently by biologists in general.

### **Types of Specimens for Dark Field**

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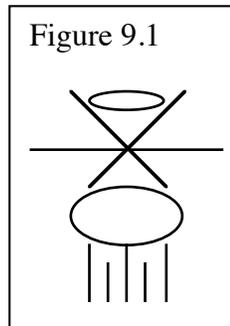
The best specimens for dark field are those that have refractive objects scattered about with empty space between them. No dark field occurs if objects are too crowded or if a thick solid specimen turns light into the microscope. Very thin histological sections can be used if unstained or if only certain components are stained, as in silver stains. Biological fluids from animals and plants, cell cultures, microbes, foods, fibers, crystals, colloids, and sub-microscopic particles are all suitable for dark field microscopy. Preparations of autoradiography and gold labeling are also suitable.

### **Dark Field Condensers**

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Dark field is the only wide field method we will study that does not strictly use Köhler illumination. In dark field microscopy, no direct light from the condenser enters the objective lens. Only light that is reflected, refracted or diffracted by the specimen enters the objective. The dark field condenser produces a circle of light. The light is at an extremely oblique angle to the surface of the slide. This oblique light comes to a focus on the specimen. It then diverges so strongly that no direct light enters the objective (figure 9.1). This type of illumination is a **hollow cone** of light. That oblique illumination used by the old microscopists to resolve N. Spencerii came only from one direction. Tilting the microscope's mirror strongly to one side produced this effect. The dark field condenser provides oblique illumination from 360 degrees around the specimen.

Figure 9.1



The numerical aperture of the condenser must be larger than the numerical aperture of the objective lens in order to prevent direct light from entering the objective lens. This is no problem for low magnification dry objectives if a 0.95 NA condenser is

used. This is a problem however for high NA objectives. Here the condenser must have a very high NA such as 1.45 and be used with an objective of no more than 1.25 NA.

### Low Magnification Dark Field Condensers

A low magnification dark field condenser can be nothing more than an ordinary bright field condenser with an **opaque disk** of the proper diameter placed in its front focal plane. The diameter of the opaque disk must be just large enough to prevent any direct light from entering the objective. Of course this means that larger disks are required for objectives of higher NA. Many microscope manufacturers produce a “universal” condenser that has one or more dark field disks that match objectives of different NA. The apparent size of an opaque disk can be made larger or smaller by raising or lowering the condenser. One disk may thus be useful for a small range of objective NAs. If the condenser has a NA greater than 0.95, better results at low magnification can be achieved if the condenser is oiled to the slide even though the objective is used dry.

You can make your own dark field opaque disk for low power objectives as follows:

- 1) Set up Köhler illumination
- 2) Observe the objective back focal plane
- 3) Adjust the aperture iris to just barely fit outside the objective aperture
- 4) Measure the aperture iris diameter
- 5) Make an opaque disk of this diameter
- 6) Place the disk below the condenser as near the condenser iris as possible
- 7) Adjust the size of the disk: if the field is gray the disk is too small, if the specimen can not be illuminated the disk is too large.

### High Numerical Aperture Dark Field Condensers

The first condenser made specifically for dark field was produced by Francis H. Wenham and George Shadbolt in 1855. This condenser used a **parabolic glass reflector** to create a hollow cone of light. Unlike a refracting condenser, a reflecting condenser does not produce chromatic aberrations and a parabolic shape minimizes spherical aberrations. The result is a more finely focused spot of light. Other dark field condenser designs emerged over the years. Figure 9.3 and Table 9.1 describe some of the more common ones.

Figure 9.2  
F.H. Wenham



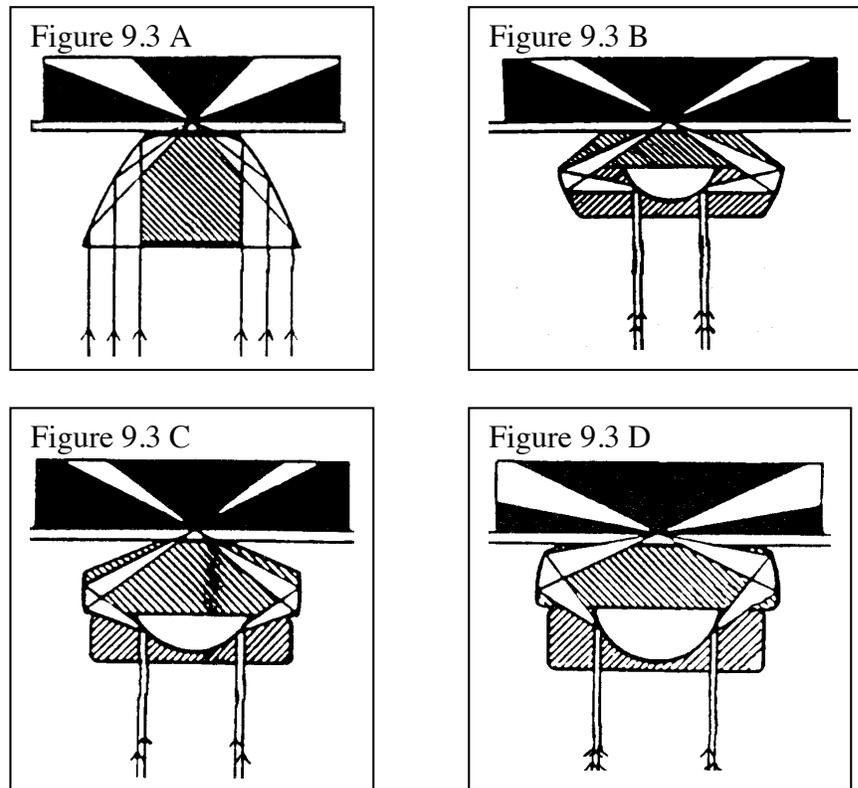


Figure 9.3 Reflecting dark field condensers:

A = Paraboloid, single reflecting type.

B = Bicentric or Bispheric, double reflecting type

C = Cardioid, one spherical and one cardioid reflecting surface

D = Spot Ring, double reflecting of maximum aperture

The need for keeping the objective NA lower than the condenser NA lead to the production of higher and higher NA condensers and to the introduction of the “**funnel stop**” for the objective lens. The funnel stop is a funnel shaped cone that is inserted into the objective lens to limit its aperture. Modern objective lenses do not use funnel stops. Instead, some high NA oil objectives have an iris diaphragm built into them. This iris is used to reduce the NA of the objective lens.

High magnification dark field condensers must always be oiled to the specimen slide. This is because the angle of incidence of light leaving the top of the condenser is much greater than the **critical angle** for glass to air; thus, no light emerges from the condenser until it has immersion oil applied to its surface.

Type	Hollow Cone NA Range	Limit NA of Objective	Number of Reflecting Surfaces	Correction	Remarks
Paraboloid	1.00 - 1.40	0.85	1 Parabolic	Partial	Must reduce oil objective's NA
Cardioid Bicentric Bispheric	1.20 - 1.33	1.05	1 Spherical & 1 Cardioid or 2 Spherical	Aplanatic	
Cassegrain	1.40 - 1.50	1.30	1 Aspheric 1 Spherical	Aplanatic	1.3 NA oil lens used at full aperture
Spot Ring Bicentric			2 Spherical		

Table 9.1 - Properties of Dark Field Condensers. Needham

### **Critical Angle In Dark Field Microscopy**

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**Critical angle** is important in dark field. The dark field condenser produces a very oblique angle of light. If this angle is greater than the critical angle at any interface, the illumination will be totally internally reflected. For this reason, the specimen's immersion medium is important. The critical angle for glass to air is 41 degrees and for glass to water it is 61 degrees. Low power dark field condensers work fine for specimens in water. A high power dark field condenser may not be useful with a water immersed specimen for the above reason. It is always best to immerse the dark field condenser to the slide with oil even for low power dark field condensers as long as the critical angle is not exceeded.

### **Methods for Dark Field Microscopy**

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Specific methods for dark field and some of the pitfalls are presented in this section.

#### **Prerequisites for dark field**

A few simple prerequisites will eliminate most of the problems met with in dark field microscopy:

- 1) Be sure your condenser has a larger numerical aperture than the highest power objective you will use.

- 2) Dark field requires a very bright light source. Remove all neutral density and colored filters from the illuminator.
- 3) Make certain your glass slides are not too thick or too thin, especially if you are using a high NA dark field condenser. High NA dark field condensers have a fixed focal length at which they optimally work. The manufacturer may recommend a slide thickness or this value may be printed on the condenser. A standard slide is 1.2 mm thick, but slides, even from the same box, vary in thickness. The slide should be no more than  $\pm 0.05$ -mm from the recommended thickness. You can determine the correct slide thickness for your condenser in the following way:
  - a) With the condenser in its uppermost position, place a slide, with one end frosted on one side, in oil contact with the condenser – frosted side facing the objective.
  - b) Examine the frosted surface with a low power (e.g. 10 X) objective.
  - c) If the image shows a bright spot with a black center, the slide is either too thick or too thin. If there is only a small bright spot the slide is the correct thickness.
  - d) If the slide is not correct, try lowering the condenser. If the black center disappears and a small bright spot remains, the slide is too thin; otherwise the slide is too thick.
  - e) Check the slide's thickness with a micrometer and repeat this process with slides of different thickness until the optimum thickness is found.

#### **Centering the dark field condenser**

If you are using a universal condenser, centering it for Köhler illumination in bright field should be adequate for use in its dark field setup. If you are using a high NA dark field condenser, use the following procedure:

- 1) Select a very low magnification (e.g. 2 X or 5X) objective.
- 2) Provide all the illumination possible.
- 3) Oil the condenser and raise it to its uppermost position under a blank slide.
- 4) If the condenser has a small central bright ring, focus the microscope on this ring and center it using the condenser centering knobs. This is a **spot ring condenser**.
- 5) If there is no ring, do the following steps:
  - a) Use a slide of the correct thickness with one end frosted on one side.
  - b) Oil the non-frosted side to the condenser.
  - c) Image the frosted side.

d) Center the bright spot of light with the condenser centering screws. Some slight adjustment with the centering screws may be necessary when shifting to the high NA objectives.

6) Assuming your slides are all the same thickness, you should not move the vertical position of the condenser.

### **Focusing in dark field at high NA**

At high magnification and high NA it may not be easy to initially focus on the specimen since the field is dark. This is especially true if the specimen is very sparse. In the case of a very dispersed specimen, a wax pencil mark on the specimen side of the slide makes a handy reference point for initial focusing. The following procedure will help in focusing:

- 1) Oil the condenser to the slide
- 2) Lower the stage (or raise the objective) and oil the top of the slide
- 3) Raise the stage (or lower the objective) until the objective and oil just touch
- 4) Look in the microscope – you should see a diffuse bright field
- 5) Focus first by increasing the distance between the slide and the objective lens (in case you are already too close) then decreasing. As you approach focus the field will become very bright then gray then black
- 6) At focus the field will be black and the specimen will shine.

### **Exercises**

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- 1) Use a sheet of lens tissue to observe the illumination emitted by a low power dark field condenser. Place the paper on the surface of the condenser and gradually raise the paper up while observing the illumination on it. Also, try placing a white card on edge on top of the condenser. Describe what you see.
- 2) Look at a cheek cell preparation by dark field. How does it compare to bright field? Can you discern the ridges on the cell surface?
- 3) Make a low magnification dark field condenser using a disk on a bright field condenser. Were you successful?
- 4) Try to determine the real structure of Amphiplura pellucida. Make a sketch. A diatom test slide is available from Carolina Biological Supply – see Appendix B.

## **A Little Review**

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Lenses focus parallel rays (direct light) produced by the condenser to a point in their back focal plane

Angled (diffracted) light from the specimen gets focused in the intermediate image plane

We can make contrast by doing one thing to the direct light and another thing to the diffracted light – such as the Rineberg filter did. And dark field completely removed the direct light

Now we are ready to do more sophisticated things with the direct and diffracted light to produce contrast: Phase Contrast

In Phase Contrast we have to think of light as a wave train with these properties:

- Speed is slower in denser media
- Trains of the same wavelength and polarization angle can interfere both positively (brighter) and negatively (darker)
- The specimen slows diffracted light by  $\sim 1/4$  wavelength relative to direct light from the same wave train – part of the wave train is diffracted and part is not
- Diffracted and direct light from one wave train can interfere in the intermediate image plane

Let's see how this works.