

# Chapter 8

## Bright Field

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This chapter collects the important information presented so far that is directly relevant to bright field microscopy. Additional information is presented on bright field technique. The chapter concludes with an interesting experiment in which colorless specimens are given color through use of a special filter that you will make. This experiment provides the grounds for a brief discussion of Abbe's theory of image formation in the light microscope.

### **Types of Specimens for Bright Field Microscopy**

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The best optics and the best instrument alignment are useless if there is no visual difference between the specimen and its surroundings or among the various parts of the specimen. Human vision is sensitive to differences in brightness (amplitude) and color (frequency) of light. To be seen in bright field, the microscopic specimen must introduce one or both of these into the uniform illuminating beam. Differential absorption and differential refraction produce contrast in bright field microscopy. Specimens that have color of their own or which can be stained are appropriate for bright field. So too are specimens that have a refractive index very different from that of the surrounding medium. Specimen contrast may, in fact, be increased by selecting a surrounding medium with a refractive index very different from that of the specimen. This is important for specimens that are colorless transparent particles such as diatoms. Most bright field specimens present some combination of absorptive and refractive contrast.

### **Methods for Bright Field**

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This section presents specific methods and common pitfalls in bright field microscopy.

#### **Condenser - Objective - Eyepiece Combinations**

Make certain that your eyepieces and objectives are matched. Compensating eyepieces require matching objectives as do CF eyepieces. For any given total magnification you should try to maximize NA. This usually means using a lower magnification eyepiece with a higher magnification objective. Remember Abbe's rule that magnification above 1000 times the objective's NA. is empty of further resolution. The NA of your condenser should be at least as large as your highest objective lens's NA. For photomicrography, your condenser should be an aplanat-achromat type.

#### **Light Source Voltage**

Adjust your illuminator to the voltage that will produce a white color of light. In the days of color film, a lamp color temperature of 3200° K was suggested as this matched the color temperature of tungsten-balanced film. Some microscopes have a built-in Photo setting on their illuminator for this temperature. Of course, colored filters in the light path

will also affect the color of the light. A blue filter is often used give the light a white appearance when the microscopist reduces the brightness by lowering the voltage of the bulb. Brightness is better adjusted by using your neutral density filters. With modern digital cameras that have white balancing capacity, setting the lamp's color temperature is not so critical. You will have to re-white balance the camera if you change the voltage. However, for consistent results it is best to run the lamp at its maximum voltage.

### Cover Glass Thickness and Cover Glass Correction Collars

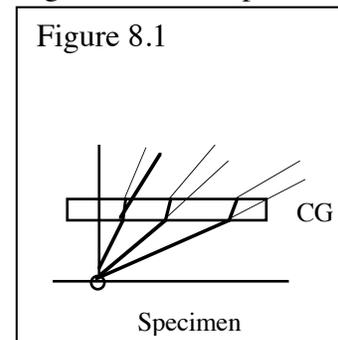
All modern objectives, both dry and immersion, have a built in spherical aberration correction for a cover glass of 0.17 mm thickness (unless the lens is labeled NCG for no cover glass). This is a # 1.5 cover glass. Prepare your slides with # 1.5 cover glasses. Some high dry objectives have built in cover glass correction collars. When used correctly, correction collars can compensate for even slight variations in cover glass thickness. Here's how to use them assuming Köhler alignment:

- 1) Open the aperture iris. This is necessary to minimize contrast in the specimen.
- 2) Focus the specimen, and observe the over all level of contrast and focus.
- 3) Note the degree of haziness over the entire field of view.
- 4) Turn the collar until the image just blurs.
- 5) Re-focus the microscope and determine if the amount of haze looks better or worse and if the over all contrast and focus is better or worse.
- 6) If better keep going in the same direction with the collar; if worse go back.
- 7) Repeat this until an image with the best possible contrast and sharpness is obtained.

If the cover glass correction collar is not used correctly, or is simply ignored, the result may be a disastrously washed out, soft, blurry image.

### Why use a Cover Glass

In the 1830's it was discovered that applying a thin slip of glass over the specimen improved the image. Why should this be so? The straight forward answer is as follows: (1) the cover glass helps cut down on irregular refraction at the surface of the specimen by providing an optically flat surface, (2) it holds the specimen flat, (3) it keeps the specimen off the objective lens, and (4) it slightly improves the angular aperture of the objective lens. Figure 8.1 illustrates how the coverglass brings highly diffracted light closer to the lens axis thus improving resolution. A coverglass introduces spherical aberrations. There is an interesting story about this discovery and about how this problem was corrected. This story is related in the narrative of Appendix A.



### **Slide Thickness**

Slide thickness is also very important. The working distance of high NA condensers is very close to 1.00 mm. If your slides are too thick (e.g., >1.2 mm) you will not be able to focus the field iris clearly and your Köhler alignment will be compromised.

### **Köhler Illumination**

The principal of Köhler illumination was covered in Chapter 2. It is the single most important preparation for bright field microscopy. It insures that the illumination consists of partially coherent light, that the angular aperture of illumination matches that of the objective lens, and that only the area of the specimen that is being viewed by the objective lens is illuminated.

### **Focusing**

Proper technique in focusing can make using the microscope a pleasure even after many hours of observation. Here are a few suggestions:

- 1) Correct adjustment of the interpupillary distance and diopter correction of each eyepiece is very important. See Chapter 1 for details on adjusting the binocular tube. When observing a specimen you should feel as though your eyes are completely relaxed, just as if you were gazing at the sky or at some far off horizon. No kidding!
- 2) If you wear glasses that correct for astigmatism, get used to using them at the microscope. The eyepieces can make diopter corrections but not astigmatism corrections.
- 3) Work with the room darkened. The only light you want in your eye is that which comes from the specimen. Everything else is a distraction.
- 4) If your microscope has a photographic or other reticule it should be in clear focus when the specimen is in focus. The reticule should seem a part of the specimen, not floating above or below the specimen.
- 5) It is harder to focus at low magnification than at high magnification because the depth of field increases as magnification decreases. Fully open the aperture iris before focusing at low magnification. While observing some small detail, go back and fourth with the focus mechanism starting with large motions and gradually reducing until you home in on focus. A few people observe a subtle "flash" in the specimen detail at best focus. Some microscopes have a flip-in magnifier for use when focusing at low magnification. Some microscopes are equipped with a focus aid or with auto focus. With these, be certain that the specimen occupies most of the field of view; otherwise, the focus mechanism may be fooled.
- 6) Many focus mechanisms tends to drift over time. Focusing a specimen then waiting a few minutes and looking again easily checks this. The mechanism should not drift out of focus using your highest magnification objective during the time it takes to make your longest photomicrographic exposures. Some stands have a way to tighten and loosen the

focus mechanism – usually by turning a ring around a focus knob or by rotating both knobs simultaneously but in opposite directions. Check your scope's manual for instructions.

### Changing Objectives

Except for the 1X and 4X objectives, **working distance** (the distance between the end of the lens and the slide) decreases as magnification increases. Never-the-less, a series of objectives from a given manufacturer will remain fairly close to focus when changing from one to another, even to the oil objectives. The idea of **parfocality** of objectives was invented by Abbe. One precaution: avoid getting oil on your dry objectives. This will make an image through them very hazy and irregular. Remember to adjust the field iris and the aperture iris when changing from one objective to another.

### Oil Immersion Technique

Each microscope manufacturer recommends a certain immersion oil (usually theirs) for their oil objectives. It is a good idea to pay attention to this recommendation. Even though most immersion oils have about the same refractive index, the color of the oil or its effect on the materials of the objective could be important. Different oils may not be miscible so you should thoroughly clean a slide when going from one type of oil to another. Some oils are fluorescent and this would be disastrous if you were doing fluorescence microscopy. Some oils will etch various plastics; you should test this if you use plastic materials to mount your specimens. Remember that the NA of a lens is partly based on the refractive index of the immersion medium ( $NA = n \sin \alpha$  where  $n$  = refractive index of the immersion medium) so increasing the oil's refractive index can increase NA and thus resolution. Immersion oils can vary in the degree to which they refract different colors of light - the phenomenon of dispersion. Most oils for general use have low dispersion. There are many fluids that have refractive indices above 1.515; however, these fluids often have a high dispersion and are not suitable for general immersion work. In my laboratory, with many microscopes of different makes, I use one type of oil for all scopes (Cargille type DF) that is compatible with all lenses. This makes switching between scopes easy and simplifies cleaning procedures.

It is very easy to get bubbles between an oil lens and the specimen slide. The bubbles will degrade image quality. Here are a few suggestions to help prevent bubbles:

- 1) Use a glass dipstick to apply oil rather than the squeeze bottles that may be provided. The squeeze bottle develops bubbles in its neck that can end up on your slide.
- 2) It is not necessary to back off on the focus before swinging in an oil immersion lens if the lens set is parfocal. Just swing the lens into position. You can even sweep the lens back and forth a few times to dislodge any trapped bubbles.
- 3) Putting a drop of oil on the objective as well as on the slide helps prevent bubbles.
- 4) Remember that an oil immersion type condenser must be oiled to the slide if the full NA of an oil immersion objective is to be achieved in transmitted light, and that the

condenser's NA must be equal to or greater than that of the objective. Do not try to oil a dry condenser (NA less than 1). It will just make a mess.

5) Oiling a condenser to a slide can be messy. Bubbles can also form between the condenser and slide. Try placing oil on the condenser with the slide removed, then put oil on both sides of the slide before placing it on the stage.

6) Check for bubbles by removing an eyepiece and looking down the tube, or better, use a phase telescope or Bertrand lens. Move the objective lens from side to side and the condenser up and down. If a bubble is present you will see its edge.

### Posture

A good chair is worth its weight in gold. Long hours at a microscope with poor seating and poor posture can make a microscopist miserable. You should sit with your back straight and both feet on the floor. Your eyes should be at exactly the same level as the eyepieces. Your head, your shoulders, and your arms should be in a comfortable position. To achieve this you need good seating. A chair with height adjustment and with tilt adjustment for the seat and the back is desirable. Good lumbar support in the chair is essential – you may need to add a small cushion behind your lower back. If your feet do not reach the floor when the chair is at the proper height for your eyes, use a footstool or other footrest. Do not let your legs dangle. Tilt the chair seat slightly forward so that you are being held toward the microscope. Now and then get up and stretch and let your eyes focus at some distant point.

### Biological Staining

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Contrast can be produced in biological samples by the addition of dyes that either selectively or generally stain the specimen. The dye absorbs certain frequencies of the illuminating light producing often strikingly beautiful results. Biological staining for microscopy is a large and complex field and will not be covered in this course. A good reference is the book by Sheehan, and Hrapchak, "Theory and Practice of Histotechnology". Table 8.1 is a sampling of biological stains.

| Component Stained           | Stain  |
|-----------------------------|--|
| General Polychromatic Stain | Hematoxylin and Eosin (H&E)                  |
| Acid-Fast                   | Kinyoun                                      |
| Acid Mucopolysaccharides    | Alcian Blue, Colloidal Iron                  |
| Alpha Cells and Pancreas    | Grimelius                                    |
| Amyloid                     | Congo Red                                    |
| Ameba                       | Wheatly, Crystal Violet                      |
| Beta Cells                  | Scott  |
| Bacteria                    | Brown-Brenn                                  |
| Calcium                     | VonKassa, Alizarin Red S                     |
| Carbohydrate                | Periodic Acid-Schiff (PAS), Alcian Blue      |
| Collagen                    | Masson Trichrome / Sirius Red                |
| DNA                         | Fulgen, Methyl Green Pyronine                |
| Elastic                     | Gomori Aldehyde Fuchsin, Verhoeff-Van Geison |

|                       |  |
|-----------------------|--|
| Fat                   | Oil Red O                                |
| Glycogen              | Periodic Acid-Schiff (PAS)               |
| Iron                  | Gomori                                   |
| Mucin                 | Mucicarmin                               |
| Muscle                | Mallory Phosphotungstic Acid-Hematoxylin |
| Pituitary             | Pearce                                   |
| Spirochetes           | Dieterle                                 |
| Argentaffin           | Fontana-Masson                           |
| Argyrophil            | Grimelius, Sevier-Munger                 |
| Basement Membrane     | Periodic Acid-Methenamine                |
| Fungus                | Gridley, Grocott                         |
| Nerve Fibers          | Bielschowsky                             |
| Pneumocystis Carinis  | Grocott, GMS                             |
| Reticulin             | Wilder, Gomori                           |
| Uric Acid             | Gomori                                   |
| Blood and Bone Marrow | Giemsa                                   |
| Myelin                | Luxol Fast Blue                          |
| Nerve Fibers          | Bodian                                   |

Table 8.1 - Biological Stains

## Optical Staining

It is possible to add contrasting colors to unstained specimens. The method was invented by Julius Rheinberg and is known as Rheinberg illumination or optical staining. The method is currently not used widely, but it provides insight into Abbe's theory of microscopy, and it produces delicately beautiful images. First the method is presented and then the theory.

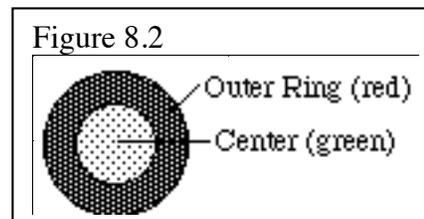
### Method for Optical Staining

A homemade Rheinberg illumination system can be constructed from colored gelatin filters and cover glasses. Figure 8.2 illustrates the arrangement of these components. The Rheinberg filter consists of two contrasting colors of gelatin filter material. One color forms an outer ring while the other color forms a solid center. The size of the center ring is very important and must be made to match a particular objective lens. Here is how:

1) Focus a specimen with the objective lens for which the filter is to be made.

2) Take out an eyepiece and while looking down the tube close the aperture iris until it just appears at the edge of the field.

3) Measure the diameter of the iris opening. To do this on an upright stand, you may have to take the condenser off the stand and turn it over to see the iris. Use a ruler to estimate the diameter of the opening. Some condensers have a millimeter scale on the iris control. For motorized condensers there is usually a readout of the aperture diameter in the



software interface. Another method is to place a clear ruler in the condenser's front focal plane and read off the diameter of the objective's back focal plane by looking down the tube with the eyepiece removed.

4) Make the center filter "slightly" larger than this. The best size must be determined by experimentation.

5) Cut the outer filter to fit snugly around the center filter. Try green for the center color and red for the outer color. It is best if the center is darker than the outer ring. This can be done by placing several center filters on top of one another or by using a black felt marker to color the center filter.

6) Place the filter pair between two cover glasses and seal the edge with wax or nail polish.

An alternative method would be to draw and color the filter in an imaging program such as ImageJ and print it on a transparent sheet using a color printer.

Place the Rheinberg filter as close to the position of the condenser iris as possible. This puts it at the front focal plane of the condenser. Some condensers have holders for accessory filters. With your filter in position, place an unstained specimen, such as cheek epithelial cells, very thin wood shavings or diatoms, on the microscope and focus. Pull out an eyepiece and while looking down the tube center your filter. The central filter should just fill the outer edge of the field of view. Put back your eyepiece and enjoy. If you used a green middle and a red rim, you will see red tinged cells on a green background.

## **Abbe's Diffraction Theory of Microscopy**

Optical Staining is a clear demonstration of Abbe's diffraction theory of microscopy. As shown in Chapter 7, small structures in the specimen act like narrow slits on the nearly parallel waves of illuminating light. These slits let some of the light pass directly through while some light is diffracted, i.e., bent from its normally straight path. The narrower the slit, (the smaller the specimen structure) the more strongly diffracted is the light. The undiffracted, direct light contributes nothing to resolution in the specimen image. It has, essentially, not been affected by the specimen and appears as a diffuse background light. The diffracted light carries the specimen information. The effect of the objective lens on diffracted light is to spread this light out over the objective's entire back focal plane. The objective lens focuses the direct light toward the center of its back focal plane.

Ernst Abbe demonstrated that a diffraction image of the specimen occurs in the objective's back focal plane. He did this by using a finely ruled grating of parallel lines as a specimen. Figures 8.3 and 8.4 illustrate this. Abbe found that at least two diffraction orders on either side of the central, undiffracted spot had to be imaged by the objective in order to completely resolve the grating. He also found that the final image could be

affected by altering the diffraction image. Assignment 1 below is a partial demonstration of Abbe's theory. See the footnote in Chapter 7 for more about Abbe's theory and Rayleigh's extension of it.

The Rheinberg filter alters the diffraction image by placing one filter color over the diffracted orders and a different color filter over the central spot. Thus the diffracted light appears as one color and the undiffracted, direct light appears as a different color.

The Rheinberg filter works even though it is placed before the specimen and not in the back focal plane of the objective. Since the filter is placed in the front focal plane of the condenser, an image of it occur at the back focal plane of the objective and this has the same effect as if the filter were actually there!



Figure 8.3 Image of a stage micrometer taken with a 20X / 0.75 objective lens. The line spacing is  $10 \mu\text{m}$ .



Figure 8.4 Diffraction pattern as seen in the objective lens back focal plane when imaging the stage micrometer shown in Figure 8.3. The central, bright 0 diffraction order results from the objective lens focusing non-diffracted, partially coherent light into its back focal plane at the focal point. The diffraction orders 1 and 2 result from the objective lens focusing light that is diffracted by the micrometer into the intermediate image plane which results in this pattern (diffraction pattern) appearing in the back focal plane. The pattern results from the interference of overlapping waves generated at the spaces in the micrometer. If you would like to know more about how this pattern is formed, read about Huygens wavelets (Pluta vol. 1).

As previously mentioned, interactions other than diffraction are occurring between the specimen and the illuminating light that contribute to contrast. These include differential absorption of light by the specimen and refraction at boundaries in the specimen where refractive index changes. However, for a colorless specimen whose refractive index is nearly equal to that of glass, diffraction is the main contributor to the image. Optical staining using Rheinberg illumination offers one form of contrast for this type of specimen.

It should be clear that diffraction alone can not make structures visible. Some method of producing contrast (such as our Rheinberg filter) must also exist. And what methods there are! The rest of this course is an exploration of these methods.

## Exercises

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1) Use a stage micrometer to observe diffraction in the objective's back focal plane (OBFP). Place the micrometer on the microscope and focus using a 20X lens. Remove the condenser. Close the field iris all the way and observe the objective's back focal plane using a Bertrand lens or phase telescope or foil with pinhole over the empty eyepiece tube. You may have to make the light as bright as possible. Try rotating the micrometer and observe what happens to the diffraction pattern. What happens if you substitute a 10X lens? Replace the micrometer with a normal tissue slide and observe the OBFP. Why is there no distinct diffraction pattern?

2) Observe the diatom *Pleurostigma* using an oil immersion objective and the condenser oiled to the slide. Remove an eyepiece and observe the objective's back focal plane. Close the condenser aperture. A six-sided array of spots should appear surrounding a bright central spot. Each spot will be blue towards the center of the field and red towards the edge. This is the diffraction image caused by the diatom's hexagonal structure. Usually only one set of six spots can be seen. This is the first order diffraction image. A second set of spots would occur outside the first set. At least two orders of spots must be collected by the objective to completely resolve the diatom's structure. What happens as you open the condenser iris? What condenser setting produces the best image of *Pleurostigma*? Why are the spots blue toward the field center and red toward the edge? (By the way, there are 44,000 dots per inch in *Pleurostigma*'s frustule).

3) Make a Rheinberg filter for your microscope. Try using an image processing or drawing program to make a number of sizes and color combinations of filters and print them on a clear, colorless, transparent material. Make the center of the filter darker than the outer part to help reduce the intensity of the direct light. Try your filters out on some cheek epithelial cells. What results did you get?