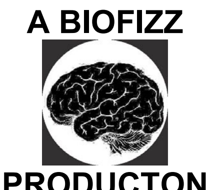
	SKILLS CENTER STANDARD OPERATING PROCEDURE	A BIOFIZZ  PRODUCTON
Light Microscopy (Bright Field) Module Hours: 3.0	**Effective Date: 05/23** **PRQs:** - **Skills Center Safety**	**Revision 1.0** **Author: Beiyi Xu** **Checked by Editor: M. Stowell**

Background

Most cells are small relative to the ability of the human eye to see detail (~0.2 mm). Magnifying them is an obvious way to solve this problem, but getting enough magnification to be useful is not a trivial task. Moreover, most cells are transparent, so even when you view them through a good magnifier, you don't see much. This situation has led to the development of many technologies to deliver clearer images with meaningful contrast. The simplest method uses two lenses in tandem (a "compound microscope") to achieve the needed magnification. Carefully controlled illumination serves both to minimize the damage that bright light can do to a living cell and to give the best possible display of detail. This method is called "bright field microscopy." Other methods for developing contrast include: 1) a "phase microscope", in which the illuminating light is split into two beams, one that is altered by material in the sample and one that is not, then the beams are combing to allow interference between them to generate contrast; 2) a "fluorescence microscope" which uses one color of light to stimulate a dye that can emit a different color and filters to block out all light except that which has been emitted by the dye. 3) Several other kinds of microscopy are available, such as one that uses polarized light, but these are more specialized techniques, not covered in our center.

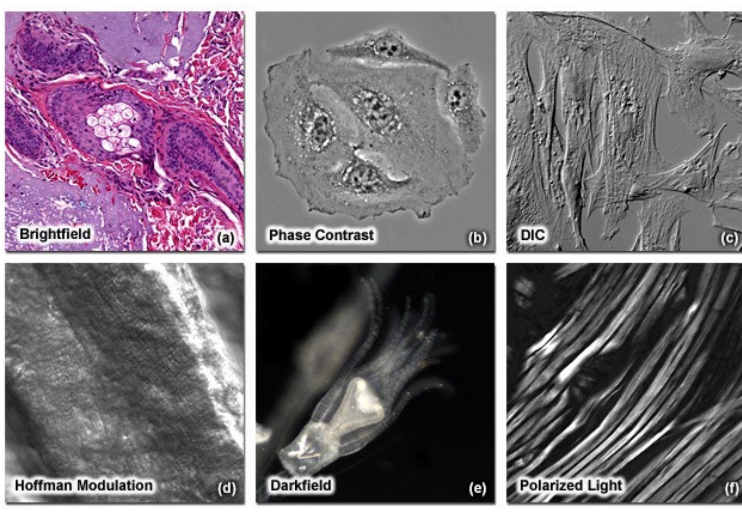


Figure 1. Examples of different techniques of microcopy.[1]

Brightfield light microscopy is a fundamental tool used by scientists to observe and analyze a variety of biological specimens. This technique can display the internal structures of cells and tissues by using a bright light source to illuminate the sample and variation in light absorption by the specimen to generate contrast. The technique is used in many fields of biology, including microbiology, cytology, and histology. The

method usually employs a compound microscope, which includes an objective lens, which forms an image of the light that has interacted with the sample and either an “ocular” lens to view that image and give additional magnification or a “projective” lens that projects the image onto a detector, such as film or an electronic camera. Bright field microscopy is one of the most used techniques for examining biological specimens. This is because it is a simple, fast, and low-cost method that can be used to study a wide range of samples, including live or dead cells, tissue sections, and microorganisms.

One of the main limitations of brightfield microscopy is that it only provides a two-dimensional image of the specimen. This can limit the ability to accurately determine the three-dimensional structure of the sample. Other limitations include the low resolution (a result of the nature of light and the lenses used to form an image) and contrast (many samples don’t absorb light, unless you stain them with dyes). There are several variations of brightfield microscopy that can be used to overcome some of the limitations of the technique. For example, phase contrast microscopy enhances the contrast of transparent samples by amplifying the differences in refractive index, while

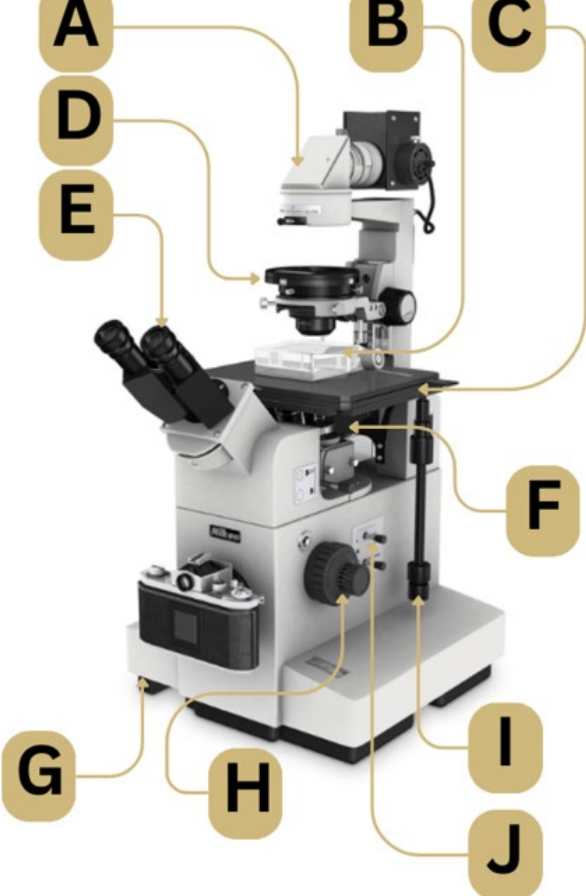


Figure 2. Nikon Diaphot-TMD Microscope[3]. (A) labels the illuminator. (B) labels the sample. (C) labels the stage. (D) labels the condenser lens. (E) labels the eyepiece. (F) labels the objective lens. (G) labels the brightness control which is hidden. (H) labels the focus knob. (I) labels the stage controls. (J) labels the optical path knob.

differential interference contrast microscopy provides a 3D-looking image of the specimen.

To perform brightfield microscopy, several pieces of equipment are required, including a microscope, a light source, and a sample preparation station. The microscope typically has multiple objective lenses with varying magnification powers. The light source should be bright enough to illuminate the sample and should be positioned properly for optimal illumination. The sample preparation station may include tools such as slides, cover slips, and staining solutions.

In summary, brightfield light microscopy is a widely used technique for the visualization and analysis of biological specimens. While it has certain limitations, such as limited resolution and contrast, it remains a valuable tool in the scientific community due to its ease of use and low cost. By understanding the basic principles and equipment necessary for brightfield microscopy, scientists can accurately observe and analyze a wide range of biological samples. [2,3]

1. **Purpose**
   - 1.1. This Standard Operating Procedure (SOP) outlines the procedure for using a bright field light microscope to visualize and analyze samples.
2. **Scope**
   - 2.1. This SOP is applicable to all laboratory personnel who are involved in using the bright field light microscope for sample visualization and analysis.
3. **Responsibility**
   - 3.1. It is the responsibility of the user to understand and perform the procedure described in this document.
   - 3.2. It is the responsibility of the user performing the procedure to fully document any deviations from the written procedure.
   - 3.3. It is the responsibility of the user to become trained on the procedure.
4. **Definitions**
   - 4.1. Brightfield microscopy: A technique in which light is transmitted through a sample, and the image is observed against a bright background.
   - 4.2. Objective lens: The lens closest to the sample that forms the initial image of the sample.

4.3. Condenser lens: The lens responsible for focusing the illuminating light onto the sample.
4.4. Numerical aperture (NA): A measure of the ability of the objective lens to capture light and resolve fine details of a sample. It is the sine of the angle between the optic axis and the most widely deflected light that the objective can capture, multiplied by the refractive index of the material in front of the objective lens (1 for air and ~1.55 for lenses that use oil).
4.5. Magnification: The degree to which an image is enlarged, typically measured as the ratio of the size of the image to the size of the actual object.
4.6. Resolution: The ability to distinguish two closely spaced objects as separate entities.
4.7. Field of view: The area of the sample that can be observed at one time through the microscope.
4.8. Working distance: The distance between the front of the objective lens and the sample.
4.9. Parfocal: The property of a microscope where a sample stays roughly in focus when switching between objective lenses of different magnification.
4.10. Köhler illumination: A technique for ensuring optimal image resolution and even illumination of a sample, achieved by adjusting the focus, aperture, and field diaphragms of the microscope.

5. Materials/Equipment

5.1. Bright field light microscope
  5.1.1. Nikon Diaphot-TMD[4]
5.2. Prepared slides
5.3. Calibration Slide
5.4. Digital Phone
5.5. Kimwipes
5.6. Gloves

6. Procedures

6.1. Set up your brightfield microscope with the appropriate objective lens.
6.2. Place your selected slide on the microscope's stage and secure it in place.
  6.2.1. The microscope's main components include the eyepiece, which you look through, and the objective lenses, which magnify the sample.

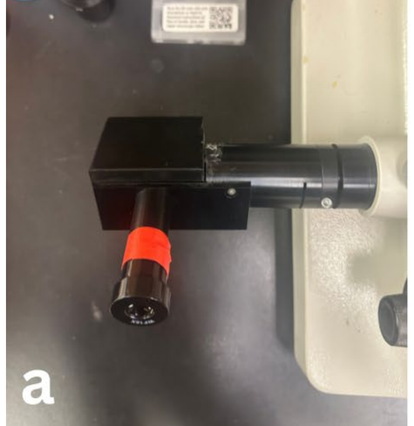
6.2.2. For this example, Sample Slide 1: Pine Leaf, c.s. will be used.

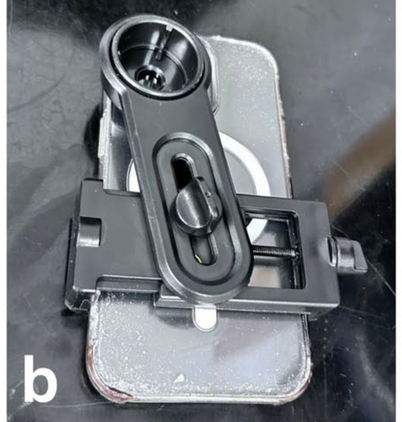
6.3. Adjust the microscope's focus by gently turning the focus knob to bring the cells into sharp focus. This ensures that you can see the cells clearly for observation.

6.4. Look through the microscope's eyepiece to view the cells on the slide.

6.5. Use the eyepiece and objective lenses to adjust the magnification for a closer examination of the cells.

6.6. Observe and make any relevant notes about the sample's appearance.





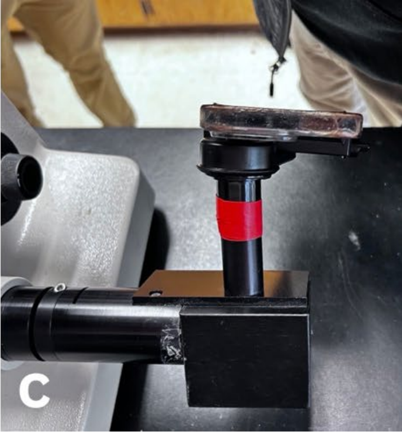


Figure 3. (a) shows the angled adapter connected to the left side of the microscope. (b) shows the phone attachment correctly placed on a smartphone. The camera lense attachment is fit on the primary sensor. (c) shows the camera lense attachment correctly attached to the angled adapter on the microscope.

6.7. Stretch out the clamp on the phone attachment and place your smartphone into it. Loosen the screw on the clamp to accommodate your phone.

6.7.1. Check that your phone's primary camera is positioned correctly within the attachment.

6.7.2. Ensure you have not switched to a different camera lens (e.g., wide-angle or telephoto). The primary camera provides the most accurate representation of the microscope's field of view. Ensure that before mounting the phone, the correct sensor is being used via waving your hand over the camera lense attachment ring.

6.8. Align the center hole on the phone attachment perfectly with your phone's primary camera lens.
  6.8.1. Attach the adapter to the angled adapter on the right of the microscope.
  6.8.2. Double-check to ensure your phone is tightly held by the clamp, and the adapter is stably attached to the adapter of the microscope.

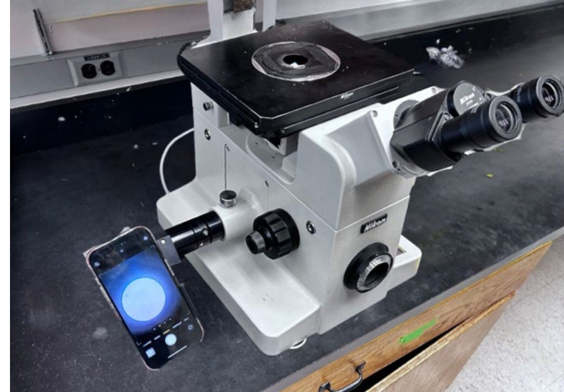


Figure 4. A smart phone correctly attached and capturing an image from the microscope.

6.9. Choose one of the prepared slides with cells for your analysis.
  6.9.1. For this example, Pine Leaf, c.s. will continue to be used.
6.10. Using your smartphone, open the camera app and access the phone attachment. Ensure the camera is focused.
  6.10.1. Ensure that the Optical path change-over knob is set to Bi/Photo and CINE/Photo. Both knobs should be pulled out and light should come out of the angled clamp.
6.11. Capture an image of the cells you wish to measure.
6.12. Capture an image of the calibration slide for reference.

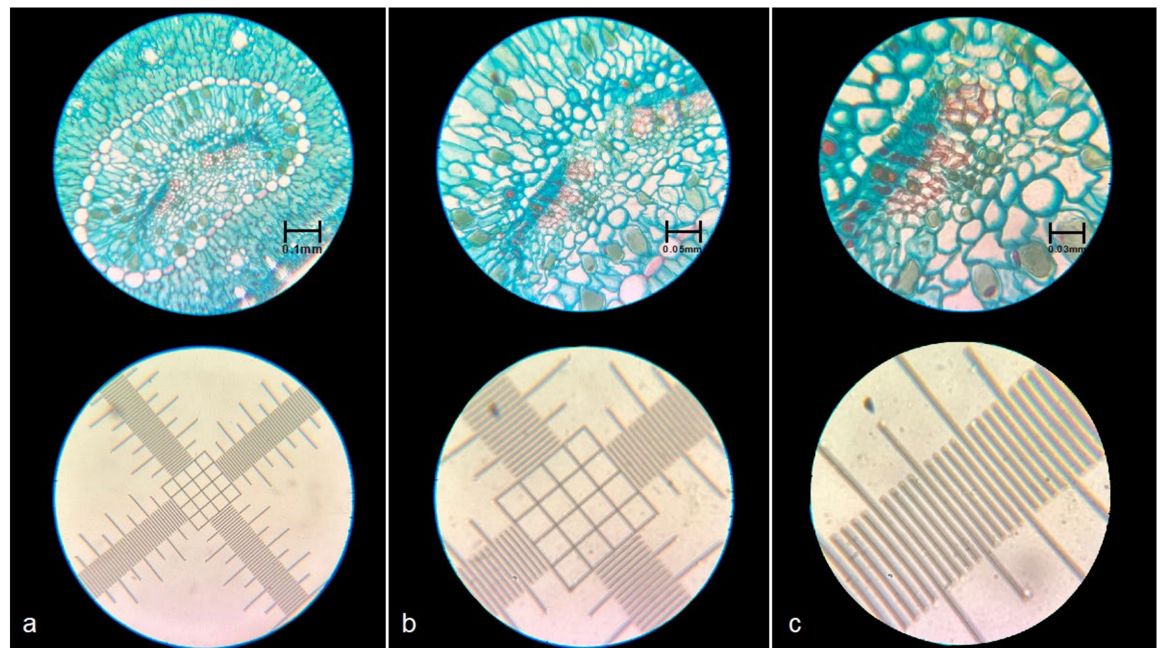


Figure 5. (a) shows the captured image of the pine leaf c.s. prepared slide along with the calibration slide at a 10x objective lens. (b) shows the captured image of the pine leaf c.s. prepared slide along with the calibration slide at a 20x objective lens. (c) shows the captured image of the pine leaf c.s. prepared slide along with the calibration slide at a 40x objective lens.

6.12.1. Do not change the focal lenses or adjust the digital camera between 6.14 and 6.15.

6.12.2. Transfer the captured images to a computer or image analysis software.

6.12.3. Overlay the images of the calibration slide and cell slide to ensure accurate measurements.

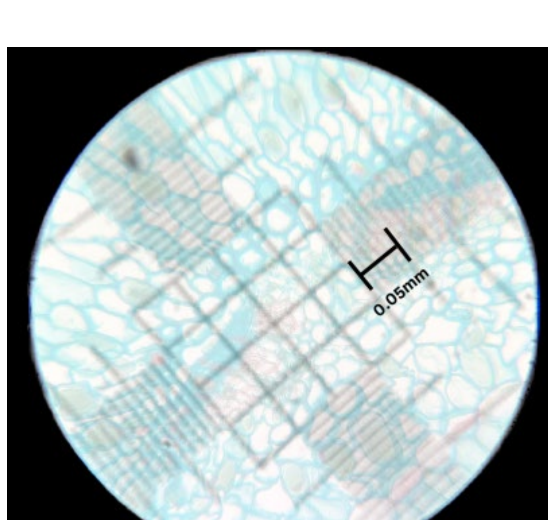


Figure 6. Calibration slide overlayed on the prepared pine leaf c.s. slide. Both are captured by a 20x objective lens. Calibration slide was set to 50% transparency using free software Canva. Lines were drawn by comparing with calibration slide (1 DIV = 0.01mm).

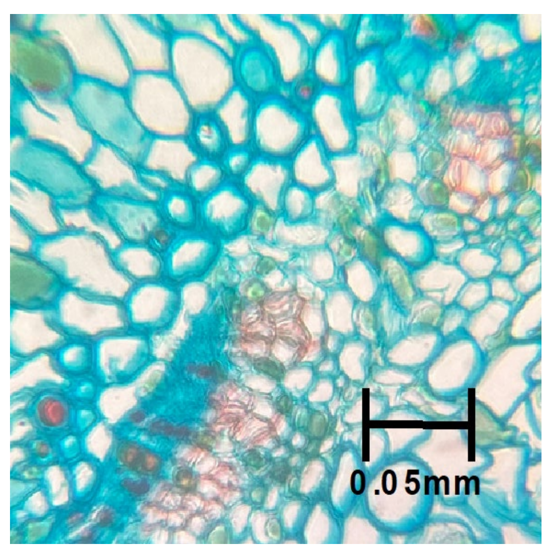


Figure 7. Post processed image of prepared pine leaf c.s. slide at 20x objective that has been calibrated with a calibration slide. Cells appear to be from 0.01mm to 0.05mm in diameter.

6.12.4. Use the known scale from the calibration slide for reference.

6.12.5. Record the measured diameters of the cells by comparing the pictures.

6.12.5.1. Document any additional observations or relevant information.

6.13. Use the data to draw conclusions about the size and variability of the cells you measured.

6.13.1. Compare your findings with any relevant research or expected cell sizes.

6.14. Remove the slide from the microscope stage.

6.15. Carefully detach your phone from the attachment.

6.16. Power off the microscope and return all equipment to its designated storage.

7. Troubleshooting

7.1. If the sample is not visible, check the following:

7.1.1. Ensure that the light source is turned on and functioning properly.
7.1.2. Check the alignment of the microscope components to ensure proper functioning.
7.1.3. Adjust the focus and light intensity as necessary.

7.2. If your measurements are inaccurate or the field of view appears distorted while taking a picture.
7.2.1. Verify that you are using the primary camera without switching lenses.
7.2.2. Confirm that the phone attachment is correctly aligned with the primary camera, and that the screw is tightened securely.
7.2.3. Recheck the camera settings on your smartphone to ensure you are using the primary camera.
7.2.4. Ensure that the Optical path change-over knob is set to Bi/Photo and CINE/Photo. Both knobs should be pulled out and light should come out of the angled clamp.

8. References

8.1. Carl Zeiss. Education in Microscopy and Digital Imaging. (2023).
8.2. Douglas B. Murphy & Michael W. Davidson. *Fundamentals of Light Microscopy and Electronic Imaging*. (2012).
8.3. Nikon's Museum of Microscopy. *Diaphot TMD Inverted Microscope*. (2023).
8.4. Nippon Kogaku K.K. *Nikon Inverted Microscope DIAPHOT-TMD Instructions*. (1980).

9. Module Mastery Task (MMT)

9.1. Describe the conditions under which you should choose a higher magnification objective lens versus a lower magnification lens. Explain how adjusting the light source can improve visibility in brightfield microscopy. Use your reasoning to choose the best objective slide necessary for the SOP.
9.2. Capture images of cells on three different prepared slides along with the calibration slide using the brightfield microscope and the attached smartphone. Ensure that the captured images are clear.
9.3. Measure the diameters of individual cells in each captured image via post processing the image. Utilize appropriate image analysis tools and techniques to

perform these measurements accurately. Create figures similar to (SOP Figure 7) to analyze.

9.4. Research literature values for the typical sizes of the cells you examined in each slide (e.g., pine leaf cells, onion epidermis cells, human blood cells). Provide references to the sources of this information. Compare your measured cell diameters to the literature values for each slide.

9.5. Explain any significant variations between your measured cell sizes and the literature values. Offer a brief description of the potential reasons behind these differences, considering the limitations of brightfield microscopy.

9.6. Write a concise conclusion that encompasses the following key elements:

- 9.6.1. Restate the purpose of the Module Mastery Task (i.e., the objectives you were expected to achieve).
- 9.6.2. Summarize the results and findings of your brightfield microscopy work, including any differences between your measurements and literature values.
- 9.6.3. Describe the most significant lessons or insights you gained from completing the tasks.
- 9.6.4. Explain how you intend to apply the knowledge and skills acquired during this module in your academic or professional pursuits.

9.7. Submit

- 9.7.1. Compile your responses to all mastery tasks into a comprehensive report that includes the measurements, comparisons, and analysis.
- 9.7.2. Attach the captured images, properly labeled, and organized.
- 9.7.3. Submit your report, images, and any additional documentation as per the guidelines provided by your instructor or proctor for review.