

## Zeiss Axiophot Microscope User Guide

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If you find a mistake or have suggestions how to improve this guide, please contact Stan Vitha ([vitha@mic.tamu.edu](mailto:vitha@mic.tamu.edu))

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### **Acknowledgment of the use of MIC facilities**

MIC guidelines mandate that the use of the microscope must be acknowledged in any publication (including web pages): **“The use of the Zeiss Axiophot microscope in the Microscopy and Imaging Center at Texas A&M University is acknowledged.”**

Users are also required to file a copy of any relevant publication containing the acknowledgment with the MCF administrative office.

### **Mercury Lamp Precautions**

- The lamp emits strong UV and visible radiation. Do not look into the source or disassemble the lamp housing. **DO NOT LOOK INTO THE OCULARS WHEN MOVING TO A DIFFERENT FLUORESCENCE FILTER. THE EPI-POLARIZING FILER SET (Slider #2) WILL REFLECT UV TO YOUR EYES.**
- Keeping track of mercury lamp usage is vital. Make sure to record the used time and the total hours accumulated usage.
- Mercury lamp lifetime is rated at 100 hr. If used beyond that point, risk of explosion and mercury contamination of the room sharply increases. **Do not turn the lamp on if the lamp reached its expected lifetime!**
- Frequent switching ON/OFF shortens the mercury lamp's life considerably. It is better to leave it on if the next user is going to need it within 1-2 hours.
- After turned on, it takes ~ 15 min for the lamp to reach full brightness.
- Lamp must be ON for at least 30 min before it can be switched OFF.
- After the lamp has been switched OFF, it must cool down (at least 30 min) before it may be switched ON again.

### **If you use oil immersion objectives, read this!**

Because the dry 40x objective has a very small working distance, it will get contaminated with immersion oil if there is some on the slide. Therefore, if you are using the 63x or 100x oil immersion objectives and want to switch to the dry 10x, you must turn the objective turret in such a direction to avoid swinging the 40x lens through the oil. You cannot use the 40x objective for observation at all, unless the slide has been cleaned and all oil removed from the coverslip.

### **Turning the system ON:**

1. Sign-in, fill out the instrument time sheet.
2. Take the protective cover off the microscope.
3. If planning to use fluorescence, turn ON the mercury lamp power. **Since the power spike from the power supply could damage other equipment, it must be turned on before anything else.**
4. Turn on the motorized focus control box.
5. Turn on the halogen lamp
6. If you plan to take pictures, start the computer and turn on the monochrome camera. The color camera does not have a power switch, it is on as long as the computer is on.

### **Turning OFF:**

1. Remove the specimen and turn off the focus control box.
2. Turn the camera OFF.
3. If oil immersion was used, clean the objectives (see detailed instructions below) and the stage.
4. Change to the smallest magnification (2.5x or 10x objective)
5. Transfer/save your images.
6. If nobody is scheduled to use fluorescence after you, turn OFF the computer, then the mercury lamp power supply and record the usage in the logbook.
7. Allow the mercury lamp to cool down for ~ 10 min, then replace the plastic dust cover. Protecting the optics from dust is very important – dust is the biggest enemy of your images, dirty optics produces blurry pictures.
8. Clean after yourself. Wipe the working area with wet paper towel, properly dispose of all used slides/coverslips, material, etc.

### **Cleaning oil immersion objectives**

1. Using clean lens paper (e.g. Fisher 11-996) gently blot off the oil from the lens. **Do NOT drag the paper across the lens, just dab off the oil.** The front lens of the objective is very delicate and must be protected from scratching.
2. Lock the objective in retracted position
3. Thorough cleaning of the objectives will be performed periodically by the MIC staff.

## Using the Microscope

1. When switching between objectives, hold on the collar of the nosepiece; **NEVER** turn the nosepiece turret by grabbing the objectives. The objectives are precision-mounted and this force could disturb their alignment.
2. With the lowest-magnification objective engaged, place the slide on the stage and locate the specimen. Select the proper filter set or other appropriate filter cube and adjust the illumination as necessary.
3. The oil immersion objectives are kept in the retracted position. In this position you will not be able to focus. To use, set them to the fully extended position.

### For those who did not pay attention:

After using oil immersion, you cannot switch back to the 40x dry objective to view the same area, because the oil would get on the lens! You could use the 10x objective, but to get there, you must turn the objective turret in the direction that will avoid contaminating the 40x lens.

### Adjusting the oculars for optimal viewing

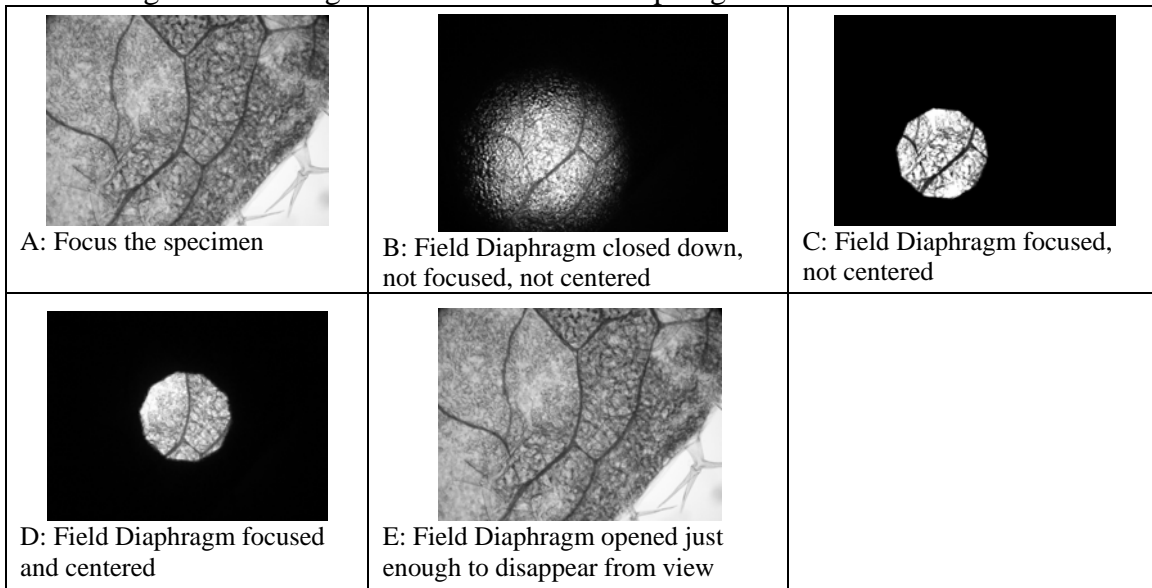
1. Adjust the interpupillary distance of the binoculars so that you can see with both eyes
2. Set both oculars to "0" focal correction (see scale on the side of the ocular tube)
3. Use the microscope focus knob to bring a specimen in focus for your left eye.
4. Now, take your hand off the focus knob. Turning the ocular focus correction, bring the specimen in focus for your right eye.

## Setting up Köhler illumination

The most overlooked component of a modern microscope is the condenser. It is a lens system that focuses the illuminating beam on the specimen and limits the resolving power of most microscopic applications. Kohler illumination produces an even field of illumination by focusing the plane of the field-limiting aperture onto the specimen plane. The optical corrections made in the objectives lenses are designed to work best with Kohler illumination. **A few seconds spent properly adjusting the condenser will greatly improve the resolving power of the microscope and is absolutely necessary for many of the more sophisticated techniques.**

1. Place specimen on the stage, and turn on the transmitted light.
2. Focus the specimen (Fig. 2A). From now on, do not touch the stage focus knob.
3. Completely open the condenser Aperture Diaphragm and completely close the Field Diaphragm (Fig. 2B). (Be sure you've correctly identified the diaphragms or you won't really be setting up Kohler illumination).
4. Focus the edges of the field diaphragm to the blue fringe, by cranking the condenser up or down using the condenser focus knob. Double check that the specimen and the field-limiting aperture are in focus simultaneously (Fig. 2C).
5. If the Field Diaphragm is off center, use the two adjusting screws to center the condenser so that the diaphragm image is exactly centered in the field of view (Fig. 2D).
6. Open the Field Diaphragm until its shadow just disappears from the field of view (Fig. 2E). Opening the Field Diaphragm more than that causes extra glare and decreases image contrast.
7. Adjust the Aperture Diaphragm so that the condenser aperture is ~ 80% of the N.A. of that on the objective. The condenser has a NA scale from 0.9 NA down to 0.1. So for a 0.5 NA objective the condenser NA should be ~0.4. For best image, the Aperture Diaphragm should be closed down just far enough to remove the glare from important portions of the specimen and to get reasonable contrast. Do not use it to cut down light intensity; this should be done by adjusting the voltage or with a neutral density filter.

**Figure 1:** Focusing and centering the condenser Field Diaphragm



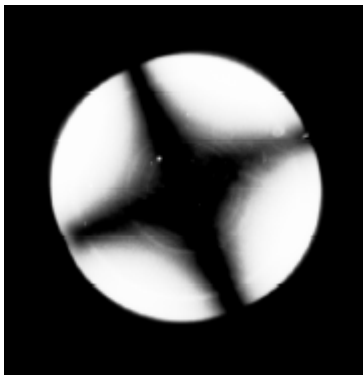
## Transmitted-light observation

### Brightfield

1. The condenser should be in position “H”
2. Make sure the Analyzer slider (= polarizer above the nosepiece, on the right side) is pulled out.
3. For maximum light intensity, also swing out the Polarizer below the condenser out of the light path.
4. Set up Köhler illumination
5. Adjust the oculars for optimal viewing

### Polarization

1. Perform setup as for brightfield, including Köhler illumination
2. Make sure there is no DIC prism in a slot above the objective.
3. Remove the specimen and engage both the Polarizer (below the condenser) and the Analyzer (above the nosepiece). The field should be very dark. If it is not, rotate the Polarizer for maximum extinction (the darkest image) –the polarizer should click into position.
4. Engage the Bertrand lens (“Ph” position on the Optovar) and look at the back focal plane of the objective. It should be uniformly dark, or, with higher-NA objectives, will show the Maltese cross pattern (Fig. 2) aligned with the vertical and horizontal axes. Close the condenser aperture so that only the central dark area of the Maltese cross is visible.



**Figure 2: Pattern at the back focal plane of a high-NA objective with crossed polars.** Note that in this picture, the cross is not aligned with the vertical or horizontal axes, because the polarizer and analyzer were installed with incorrect rotation.

5. Disengage the Bertrand lens (switch to “1.25x” position on the Optovar).
6. View your specimen. The stage can be rotated after loosening the knob on its front.

## Differential Interference Contrast (Nomarski)

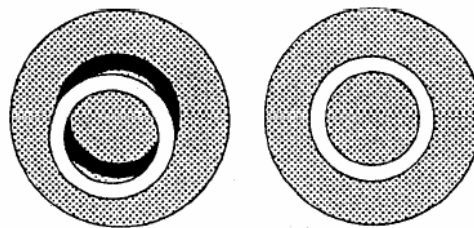
This mode is possible with the 10x, 40x, 63x, and 100x lenses. It can be used without a fluorescence filter set (dummy filter holder) or with most fluorescence filter sets.

1. Perform setup for brightfield, including Köhler illumination.
2. Make sure the Polarizer below the condenser is engaged and in correct orientation (the lever towards you in the click position), and that the Analyzer slider above the objective is engaged (pushed in).
3. According to the numerical aperture of the objective used, select the proper position on the condenser: There are two positions, for objectives with NA up to 0.4, and for NA between 0.5 and 1.3 (use also for the 63x/1.4 oil immersion objective).
4. Fully open the condenser aperture.
5. Insert a Nomarski prism slider in the slot above the objective. Each slider is clearly marked for which objective it is to be used (the 100x slider can also be used with the 63x objective).
6. Adjustment of the image can be made by turning a knob on the Nomarski prism. If there is not enough contrast in the image, try closing the condenser aperture slightly.

## Phase Contrast

This mode is possible only with objectives marked “Ph”, i.e., the 20x, 40x, 63x, and 100x.

1. Set up as for Brightfield
2. Place your specimen on the stage
3. Turn the condenser to the appropriate Phase Contrast position, Ph2 or Ph3, corresponding to the label on the objective.
4. Engage the Bertrand lens (turning the Optovar on the right side above the nosepiece to the “Ph” position) and focus the Bertrand lens to see the phase ring at the back focal plane of the objective.
5. The dark phase ring and the bright condenser annular apertures should coincide (Figure 1). If necessary, align the condenser annular aperture with the dark phase ring, using two small hex keys inserted in the slots on the back of the condenser turret.
6. Disengage the Bertrand lens by turning the Optovar to the 1.25x position.
7. The alignment must be re-checked every time the condenser or the objective turret has been turned, or if new specimen is viewed.



**Figure 2. Alignment for phase contrast observation, view of the back focal plane of the objective.** Left: condenser phase annulus not aligned with the dark phase ring of the objective Right: correct alignment, the two rings coincide.

## Darkfield:

Currently limited to the 10x objective. It uses the annular aperture Ph3 to illuminate the specimen.

1. Set up as for brightfield, using the 10x objective
2. Turn the condenser to the Ph3 position

## Epiillumination techniques:

These utilize either the mercury lamp (for epifluorescence) or the upper halogen lamp (for reflected modes). Switch between the halogen and mercury by flipping the lever in between the two housing in the back of the microscope. **In order to get the specimen properly illuminated, the auxiliary lens positioned behind the fluorescent filter slider should always be used.** The only exception is when using the stand-alone mirror cube “H”, which already has the auxiliary lens built in. For all other observations, including fluorescence, the auxiliary lens must be present.

### Reflected brightfield.

Possible with all objectives; however, with the 2.5x objective some hot spots and internal reflections are visible. There are two filter cubes that can be used for brightfield observation; the stand-alone “H” cube, or the Polarizing filter set in the filter cube slider..

- 1) Select halogen as the light source
- 2) Select the upper halogen lamp,(switch on the right side of the scope next to the lamp voltage knob)
- 3) Insert the filter cube for brightfield observation: either the “H” cube, or use the polarizing filter set on the 3-FL filter slider. **Read the above note regarding the auxiliary lens**
- 4) Make sure the Analyzer slider is pulled out (disengaged).
- 5) Turn the lamp on using the voltage control knob
- 6) Pull out the shutter slider in the epiillumination path to a desired position (ND filter 0.6, ND filter 0.3, no filter)
- 7) Fully open the condenser aperture (push in the control lever)
- 8) Fully open the field aperture on the epiillumination condenser (push in the control lever)
- 9) Focus on the specimen

### Reflected Polarization

1. Set up as for reflected brightfield. You must use the Polarizing filter set, not the “H” cube.
2. Make sure there is no Nomarski prism in the slot above the objective.
3. Engage the Analyzer

### Reflected DIC (Nomarski)

1. Set up as for polarization
2. Insert appropriate Nomarski prism to the slot above the objective. Turn the adjustment knob on the Nomarski prism for best image.

## Epifluorescence

We have are two filter sliders, each holds three filter sets:

Slider 1:

- I. .DAPI set (ex. 365 nm, em. 420-470 nm)
- II. Texas Red set (ex. 530-585, em. 615 nm longpass)
- III. GFP set (ex. 450-490 nm, em. 500-550 nm)

Slider 2:

- I. Alexa 430 set (ex. 436 nm, em. 520 nm longpass)
- II. EPI-POLARIZATION (do not use with fluorescence)
- III. Cy3 set (ex. 546 nm, em. 590 nm longpass)

***CAUTION: DO NOT USE THE EPI-POLARIZATION SET WHEN THE MERCURY LAMP IS USED. This set does not have a barrier filter, thus both UV and visible light reflected from the specimen can enter the eyepieces. When switching from the Alexa 430 to the Cy3 set, close the fluorescence light shutter and do not look in the oculars..***

1. Use brightfield or other reflected or transmitted techniques to locate your specimen.
2. Switch to the Mercury lamp as the light source
3. Select the proper filter cube for your fluorochrome. Make sure to have the auxiliary lens in place.
4. Make sure the Analyzer slider is pulled out (disengaged), otherwise signal intensity is reduced.
5. Make sure the Nomarski prism is not inserted in the slot above the objective, since it would cause loss of fluorescence image quality.
6. Pull out the shutter slider (in the epiillumination path) to a desired position (ND filter 0.6, ND filter 0.3, no ND filter)
7. Fully open the condenser aperture (push in the control lever)
8. Fully open the field aperture on the epiillumination condenser (push in the control lever)
9. Focus on the specimen.
10. When not viewing the specimen, close the fluorescence shutter (push the shutter slider in) to minimize photobleaching of the specimen.



## Where to Save Image Files

- Create a folder with your name in “C:\Users\” folder. Create sub-folders as needed.
- When finished, copy your files onto your media (CD or Zip). Blank CD may be purchased from the microscopy center. It is recommended that you create two copies of your files to avoid data loss. Once you confirmed that the data are saved on your media, erase the files from the computer.
- This old system (Windows 98) does not recognize USB memory drives.
- Please note that the imaging computer is not connected to the internet
- Old image files will be purged periodically to free disk space.

## Storing your data

- Do not use Zip disks for long-term storage
- If using CDs or DVDs, store the disks in protective cover in a cool, dark place – the CD-burning layer is heat and light sensitive (after all this is how the data is recorded).
- Always create two copies of the data files, store them in different locations.
- Avoid using acidic markers (Sharpie) for labeling disks. Use a non-acidic archival felt-tip marker
- Most CDs and DVDs are not an archival medium; they can decay and be unreadable in few years, especially if not stored properly.

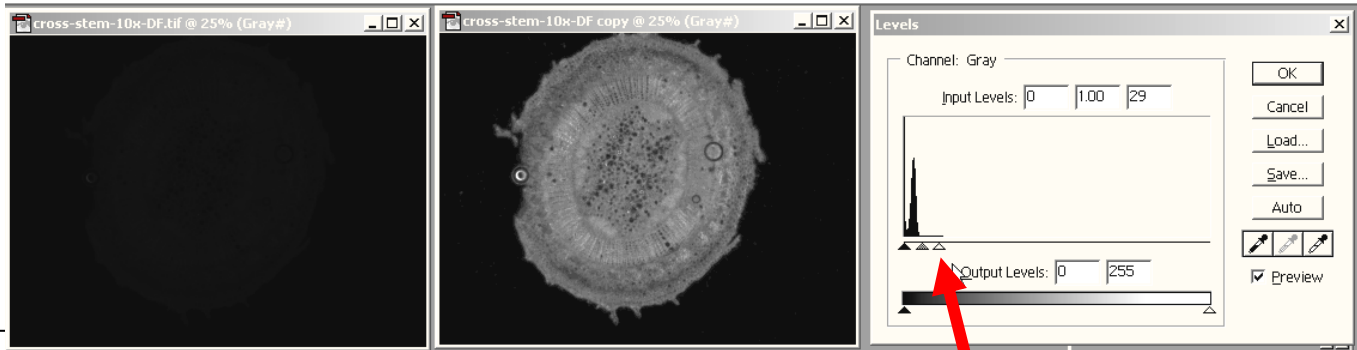
## Working with your images

Images from the monochrome camera are saved as 16-bit grayscale TIFFs. Users have recently experienced problems opening the images with Photoshop and IrfanView (<http://www.irfanview.com>), probably due to incompatible file headers. Therefore it is recommended to use ImageJ for viewing and processing the images, or use the BatchConverter plugin in ImageJ to resave files as TIFF with the correct file header. This plugin converts all files in a specified folder and saves them in a user-specified destination directory.

ImageJ is an image processing and analysis software freely downloadable from <http://rsb.info.nih.gov/ij/>. It scales the intensity values automatically upon opening the image.. This software also allows opening Metamorph .” stk” image stacks acquired with our microscope (e.g. when doing z-sectioning or time lapse imaging). These are standard multi-image TIFF files.

The files acquired and saved with the Metamorph software can be also viewed with the free “MetaPlayer” viewer (<http://www.moleculardevices.com/pages/software/metaplayer.html>). The advantage is that distance calibration (pixel size) can be shown (providing the images have been distance calibrated during acquisition).

Please note that the monochrome camera acquires images at 12-bits-per-pixel (4096 gray levels). However, the image files are saved as 16-bits per pixel (65535 gray kevels), with the image data in the first 4096 intensity levels of the 65536. Thus, if the 16-bit images are opened in **Photoshop**, they will look all black. Use the “Image- Adjust-Levels” command to lower the input max intensity value (see arrow in Fig. 3). You may need to do this twice for more precise control.



**Figure 3: Scaling grayscale intensities in Adobe Photoshop. the original image is on the left. A copy of the image is adjusted using the “Levels” command by lowering the max input intensity value (arrow).**

A very fast and convenient way of looking at many images is using the freely available IrfanView image browser (<http://www.irfanview.com>). It autoscales the intensity min and max values for display.

### Using your images in Powerpoint presentations:

Powerpoint cannot handle 16-bit images. You will have to convert your images to 8-bit first.

Recommended workflow is as follows:

1. Open your image in ImageJ or Photoshop or other image editing program, adjust the levels and/or brightness and contrast. Perform any other image adjustments necessary.
2. Convert the image to 8-bit (Image-Mode-8Bits/Channel”.
3. Resize (resample) the image to fit the Powerpoint slide. Your image should be smaller than 800 x 600 pixels. Larger files do not bring any improvement in screen resolution and inflate the Powerpoint file size.
4. Save this 8-bit image in a new file if necessary. Do not overwrite the original!
5. Copy and paste into Powerpoint

### Further training in Image Analysis and Processing:

MIC offers periodic short seminars on image processing. Please contact Stan Vitha for information. We also offer a **Light Microscopy course** each Spring. If you want to learn about various optical imaging techniques and what they can do for you, this may be the course to take.