

Zeiss Axiophot Microscope User Guide

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Acknowledgment of the use of MIC facilities

MIC guidelines mandate that the use of the MIC facility must be acknowledged in any publication (including web pages): *“The use of Texas A&M University Microscopy and Imaging Center Core Facility (RRID:SCR_022128) is acknowledged.”*

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

Turning the system ON

1. Start the Kiosk session in iLab, then fill out the instrument time sheet.
2. Take the protective cover off the microscope.
3. Turn on the motorized focus control box.
4. Turn on the halogen lamp
5. Start the computer.

Shutdown

1. Remove the specimen and turn off the focus control box.
2. If oil immersion was used, clean the objectives (see detailed instructions below) and the stage.
3. Change to the 10x objective
4. Clean the working area, put away or discard your slides/coverslips, ...
5. Transfer/save your images to a USB drive.
6. Shut the computer down
7. Finish your iLab Kiosk session.

Using the Microscope

1. When switching between objectives, hold on the collar of the nosepiece; **NEVER** turn the nosepiece turret by grabbing the objectives.
2. With the 10x objective engaged, place the slide on the stage and locate the specimen. Select the proper imaging mode (transmitted or reflected, brightfield/phase Contrast/DIC and adjust the illumination as necessary.
3. The oil immersion objectives are not installed on the turret, they are kept in the top drawer. You will need to get instructions how to properly install, use, and clean these objectives.

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.

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.

Cleaning oil immersion objectives

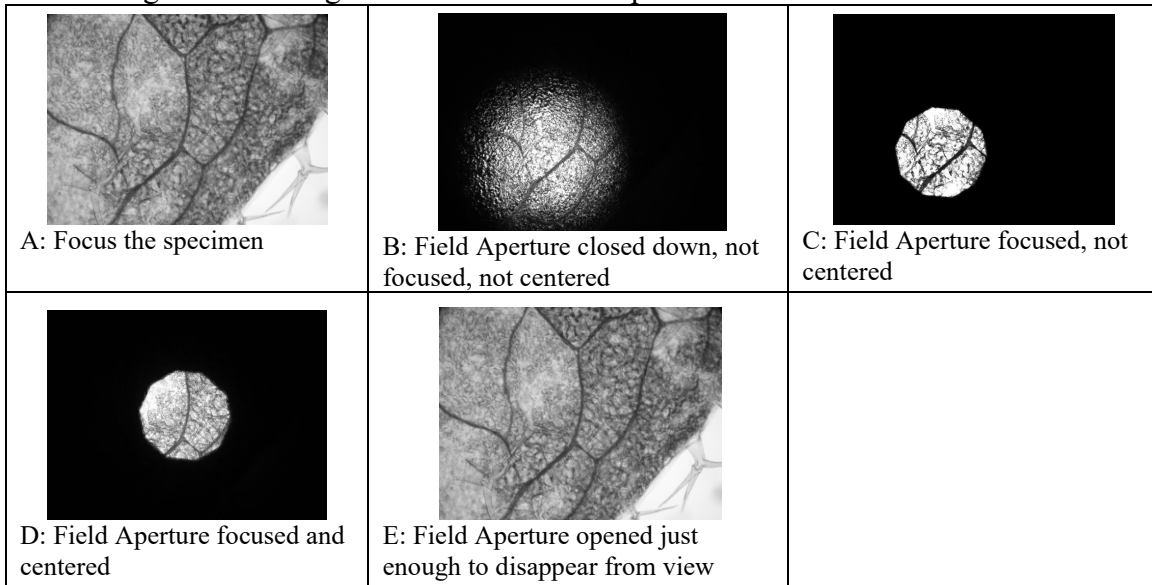
1. Use only the lens paper (e.g. Fisher 11-996). **NEVER USE KIMWIPES OR PAPER TOWELS TO CLEAN THE OBJECTIVE LENS.** Do NOT drag the paper across the lens, just blot off the oil. The front lens of the objective is very delicate and must be protected from scratching.
2. Lock the objective in retracted position
3. Thorough cleaning of the objectives will be performed periodically by the MIC staff.

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. Two major benefits of Köhler illumination: (i) even field of illumination, and (ii) dust and dirt from the illuminating optics does not show in the image. **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. Focus the specimen. From now on, do not touch the stage focus knob.
2. Open the Condenser Aperture and completely close the Field Aperture. You should see the image of the field aperture as a small circle of light projected on the specimen. (Fig. 2B)
3. Move the condenser up or down using the condenser focus knob, in order to see a focused image of the field aperture projected onto the specimen (Fig. 2C).
4. If the Field Aperture is off center, use the two adjusting screws on the condenser to center it (Fig. 2D).
5. Open the Field Aperture until its shadow just disappears from the field of view (Fig. 2E). Opening the Field Aperture more than that causes extra glare and decreases image contrast.
6. Adjust the Condenser Aperture so that the condenser aperture is $\sim 80\%$ 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 .
7. Remember that closing down the Condenser Aperture (to get more contrast) decreases the resolution.

Figure 1: Focusing and centering the condenser Field Aperture



Transmitted-light observation

The switch on the right side of the microscope, behind the lamp brightness knob, is switched to TRANS Lamp position.

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. The Optovar Turret (below the Analyzer slider) should be set to 1.25x magnification.
4. For maximum light intensity, also swing out the Polarizer below the condenser out of the light path.
5. Set up Köhler illumination
6. Adjust the oculars for optimal viewing

Polarization

Works best with the 10x dry objective.

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 (located at the bottom of the condenser) for maximum extinction (the darkest image) –the polarizer should click into position.
4. View your specimen. The stage can be rotated after loosening the knob on the front.

Differential Interference Contrast (Nomarski)

This mode is possible with the 10x, 20x, 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, but leave the condenser aperture fully open.
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 DIC position (RED label) on the condenser: There are two positions, “DIC .3-.4” for objectives with NA up to 0.4, and “DIC.5-1.3” 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. Turn the condenser to the appropriate Phase Contrast position, Ph2 or Ph3, corresponding to the label on the objective.
3. Remove the specimen from the stage or move to the clear area of the slide
4. Engage the Bertrand lens (turning the Optovar on the right side above the nosepiece to the “Ph” position) and focus the Bertrand lens (knurled ring on the lower part of the Optovar turret) 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.

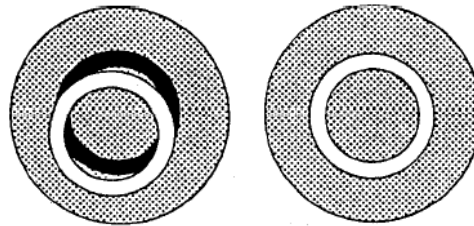


Figure 2. Alignment for phase contrast observation, view of the back focal plane of the objective. Left: condenser phase annulus not aligned with the dark phase ring of the objective Right: correct alignment, the two rings coincide.

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 the upper halogen lamp (for reflected modes). Switch between the bottom and upper halogen lamp by the switch behind the lamp brightness knob on the right side of the microscope.

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. If using the “H cube”, remove the auxiliary lens slider. The Auxiliary lens must be used if using the Fluorescence cube set with the Polarizing filter.

- 1) 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**
- 2) Make sure the Analyzer slider is pulled out (disengaged).
- 3) Turn the lamp on using the voltage control knob
- 4) Pull out the shutter slider in the epiillumination path to a desired position (ND filter 0.6, ND filter 0.3, no filter)
- 5) Fully open the condenser aperture (push in the control lever)
- 6) Fully open the field aperture on the epiillumination condenser (push in the control lever)
- 7) 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.

Capturing Images

The color camera can be controlled either by Micro-Manager or by the IC Capture software. Micro-manager allows to control both the microscope focus and the camera, while TS Image only controls the camera (but is more responsive).

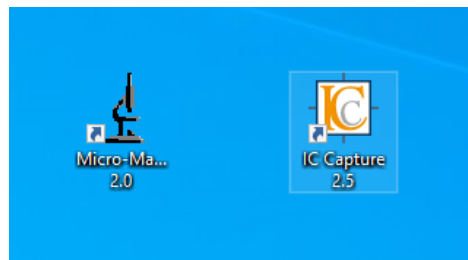
Start Micro-manager, select the default configuration file (TIS-COLOR).

For transmitted light observation, make sure the daylight balancing filter (right side of the microscope, at the bottom) is pushed in. For reflected light imaging, pull out the heat blocking filter on the epiilluminator.

To color balance the image, set up the live view, adjust gain, exposure time, then draw a rectangular ROI in the clear area of the sample that represent neutral gray/white color. Set the “WB” pre-set on, then turn it off. Cancel the ROI selection.

Your image should now be properly white balanced.

If you change lamp brightness or change filters, you may need to white-balance again.



Where to Save Image Files

- Create a folder with your name in “Documents” folder. Create sub-folders as needed.
- When finished, copy your files onto your USB drive.
- Please note that the imaging computer is not connected to the internet
- Old image files will be purged periodically to free disk space.

Viewing and processing the image files

The Micro-Manager software allows saving multi-dimensional data (time-lapse, z-stack, multi-channel) either as individual images (one tiff file for each channel, z-position, time-point) or as a single multi-image TIFF file. This multi-image TIFF file also contains all the metadata in the file header. The best way to open these images is to download and install MicroManager on your computer (<http://www.micro-manager.org>).

The multi-file data can also be opened using ImageJ or Fiji.

Storing your data

- All drives may fail. The disk on your computer is not a permanent storage solution.
- Always create two copies of the data files, store them in different locations.

Adding a scale bar to your images:

Scale bars are used for display and presentation of the images, but are not very useful for calibrating the image for measurements. For image measurements and image analysis, you do not need to have a scale bar on the image, you just need to tell your analysis software that one pixel corresponds to x.xx micrometers.

As long as you know what objective was used for acquiring the image, and providing you used the 1.25x Optovar setting on the microscope, it is very easy to add a scale bar to your existing images.

The table below lists pixel size when using the TIS color camera and a 0.6x reducing adapter, without pixel binning. Nyquist sampling is achieved for all objectives. No advantage is gained by using the 100x/1.3 oil objective instead of the 63x/1.4 oil, unless Phase Contrast is needed

Objective	pixel size	Calculated XY resolution	Calculated Z resolution	Suggested Z-step
	[μm]	[μm]	[μm]	[μm]
Plan Neofluor 2.5x/0.075	1.546	4.067	124.82	54.27
Plan Neofluor 5x/0.15	0.814	2.033	31.20	13.57
Plan Neofluor 10x/0.3	0.405	1.017	7.80	3.39
Plan Neofluor 20x/0.5	0.203	0.610	2.81	1.22
Plan Neofluor 40x/0.75	0.100	0.407	1.25	0.54
Plan Apo 63x/1.4 oil	0.064	0.218	0.54	0.24
Plan Neofluor 100x/1.3 oil	0.040	0.235	0.63	0.27

XY resolution calculated as $D=0.61*\lambda/NA$

Z-resolution calculated as $Dz=1.4*\lambda*\eta/(NA^2)$ where η is the refractive index of immersion medium, λ is the wavelength (500 nm, blue light)

Adding scale bars using ImageJ freeware

- Start ImageJ, open the image of interest.
- “Analyze-Set Scale”. Enter the pixel size and units. If you check “Global”, the spatial calibration will be applied to all non-calibrated images currently open or opened afterwards.
- “Analyze – Tools – Scale Bar” . Set the size and color of the bar, position and font size. If your image has a busy background in the bar area, you can fill the area around the bar (“Background” option in the dialog window).

