# Olympus Confocal 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)

**Contents:**



## Acknowledgment policies

MIC guidelines mandate that the use of the Olympus Confocal Microscope must be properly acknowledged in any publication (including web pages). You can use the following statement:

**"The use of the Microscopy and Imaging Center facility at Texas A&M University is acknowledged. The Olympus FV1000 confocal microscope acquisition was supported by the Office of the Vice President for Research at Texas A&M University."**

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.
- Mercury lamp lifetime is rated at either 200 or 300 hrs (see the sign on the power supply). If used beyond that point, the risk of explosion and mercury contamination of the room sharply increases. **Do not turn the lamp on if the lamp usage counter 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 (~15 min) before it may be switched ON again.

## Turning the system ON and OFF:

Please follow the steps in a separate instruction sheet located in the confocal room. This sheet is also available for download from our web site: http://microscopy.tamu.edu/instruments/light-microscopy/Olympus_confocal_startup.pdf.

**The Argon ion and HeNe laser life is shortened by frequent turning on and off. Therefore, leave these lasers ON during the day and turn it off only if you are the last user of the day.**

## 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 the 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!

1) **THE OIL IMMERSION OBJECTIVE MUST BE CLEANED BEFORE ADDING MORE OIL AND VIEWING THE NEXT SLIDE.** Otherwise the excess oil will run down the objective barrel and spill into the microscope.

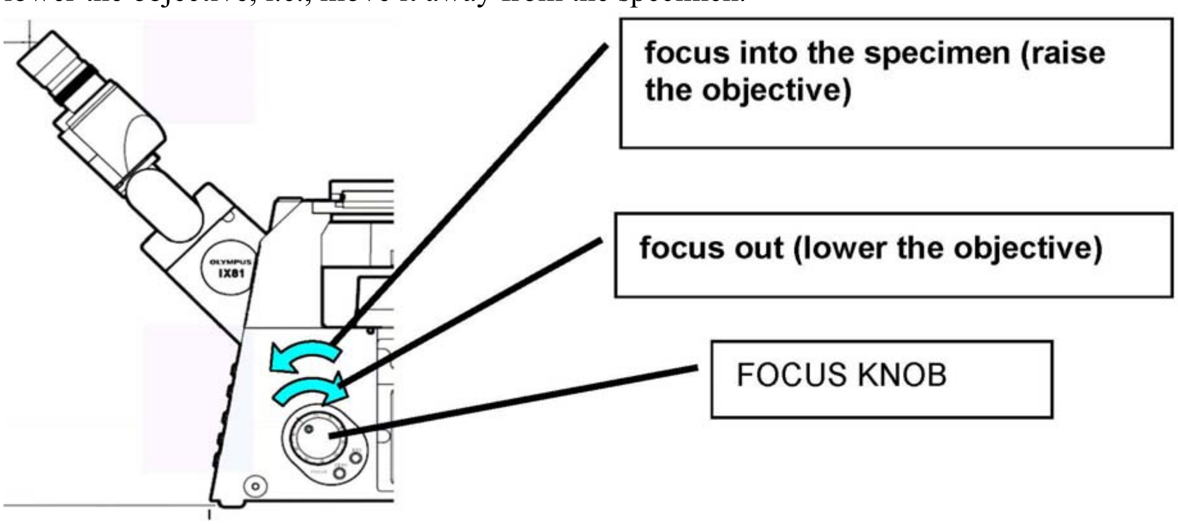
2) **BECAUSE THE DRY 20X AND 40X OBJECTIVES HAVE A VERY SMALL WORKING DISTANCE, THEY MUST NOT BE USED AFTER OIL IMMERSION OBJECTIVES UNLESS ALL OIL NAS BEEN REMOVED FROM THE COVERSLIP.** It is OK to use the dry 10x objective after the oil immersion ones, since its working distance is sufficiently large. THE CONFOCAL SOFTWARE MUST BE RUNNING - ONLY THEN IS THE MICROSCOPE SMART ENOUGH TO LOWER THE OBJECTIVES BEFORE TURNING THE OBJECTIVE NOSEPIECE TO NEW POSITION.

## Cleaning oil immersion objectives

1. NEVER USE KIMWIPES OR OTHER TISSUE PAPER TO CLEAN OBJECTIVES. **USE ONLY LENS PAPER** (e.g. Fisher 11-996)
2. Using clean lens paper 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.
3. Wipe off any oil form the objective barrel.
4. Thorough cleaning of the oil immersion objectives is performed by the MIC staff.

## Focusing

Turning the microscope focus wheel towards you will move the objectives upwards, closer towards the specimen (= focus into the specimen). Turning the focus knob away from you will lower the objective, i.e., move it away from the specimen.



# Using the Microscope

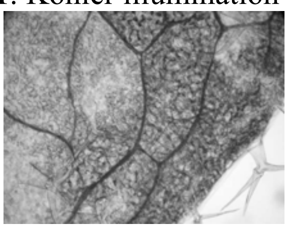
1. Turn on the needed hardware and start the confocal software. **THE CONFOCAL SOFTWARE MUST BE RUNNING BEFORE YOU PROCEED TO STEP 2.** Click on the trans-lamp button in the software. This will allow you to look at the specimen through the oculars, using transmitted light.
2. With the lowest-magnification objective (10X dry) engaged, place the slide on the stage, COVERSLIP DOWN (= towards the objective), locate the specimen and focus. If the specimen is difficult to locate, try to focus on the edge of the coverslip first.
3. Adjust Kohler illumination (see next section). THIS IS EXTREMELY IMPORTANT. IF YOU WANT TO ACQUIRE NOMARSKI (DIC) IN ADDITION TO FLUORESCENCE IMAGES, YOU MUST DO THIS TO GET GOOD IMAGES.
4. Make sure the polarizer above the condenser is engaged. Turning the knob on the Nomarski prism (below the stage and the objective nosepiece) adjust the DIC image for best image. Do not push buttons on the microscope keypad to switch to a different filter set. This will cause problems during later steps. If you do not need to visually check the fluorescence signal, click on the "Trans-lamp" button again to switch back to the confocal imaging with lasers, proceed to step 8.
5. If you need to visually inspect the fluorescence of your samples using the mercury lamp illumination, click on the "Epi-lamp" button in the software.
6. Make sure that the mechanical excitation shutter below the objective nosepiece is open. Choose appropriate filter set using the microscope keypad. The mercury lamp brightness can be adjusted by the aperture on the lamp housing.
7. When done with visual inspection of the sample, click on the "Epi-lamp" button again to switch to the confocal imaging with lasers.
8. Select your dyes from the dye database, or load the imaging conditions from an image saved previously. Adjust the detector HV and offset as needed. As a good starting point with new samples, set your HV to 700 on the fluorescence detectors, laser power to 1% (405nm) 5% (Argon-ion) 20% (HeNe lasers), scan speed (=pixel dwell time) to 8 µs, image size 512 x 512 pixels, confocal zoom 1x. The transmitted detector (TD1) will need much lower HV setting, typically around 100.
9. Start live view using the "Focus 2x" or "Focus 4x" modes and adjust the detector HV and offset to get good image. Use the Ctrl-H key to toggle between pseudocolor and saturation-warning LUT (Lookup table). The goal is to have a very dark background (some blue pixels) and a minimal amount of saturated pixels (red) in the area of interest.
10. Adjust the confocal zoom and scan size to achieve desired resolution.
11. If the HV needs to be set above 700, the images will be noisy. In order to collect more signal from the specimen, use higher numerical aperture objective if possible. Thus, it is better to use a 20x/0.75 objective at confocal zoom 2 than the 10x/0.4 objective at confocal zoom 4. Noise can be also decreased by lowering scan speed, using Kalman filtering (averaging), or increasing the laser power. In samples that are very light-sensitive, you may also need to open the CA (confocal aperture) beyond its optimum setting. This will collect more light, but decrease z-resolution. ***For very weak signals, better-quality images are obtained in the photon-counting mode.***

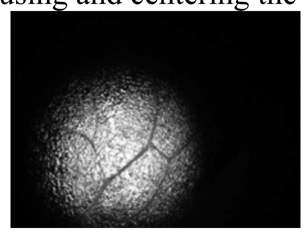
## Setting up Köhler illumination

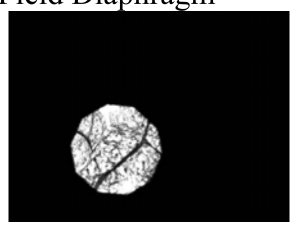
The most overlooked component of a modern microscope is the condenser. It is a lens system that focuses the illuminating beam on the specimen and limits the resolving power of most microscopic applications. Kohler illumination produces an even field of illumination by focusing the plane of the field-limiting 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.**

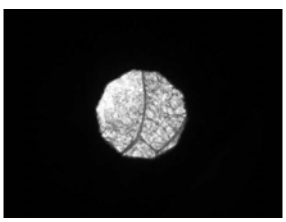
1. Place specimen on the stage, and turn on the transmitted light.
2. Focus the specimen (Fig. 1A). From now on, do not touch the stage focus knob.
3. Completely open the condenser Aperture Diaphragm and completely close the Field Diaphragm (Fig. 1B). (Be sure you've correctly identified the diaphragms or you won't really be setting up Kohler illumination).
4. Focus the edges of the field diaphragm 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. 1C).
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. 1D).
6. Open the Field Diaphragm until its shadow just disappears from the field of view (Fig. 1E). Opening the Field Diaphragm more than that causes extra glare and decreases image contrast.

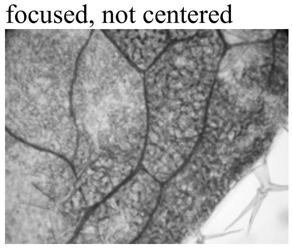
**Figure 1**: Köhler illumination - Focusing and centering the condenser Field Diaphragm


A: Focus the specimen


B: Field Diaphragm closed down, not focused, not centered


C: Field Diaphragm focused, not centered


D: Field Diaphragm focused and centered


E: Field Diaphragm opened just enough to disappear from view

## Setting z-stack start and end positions

Turning the microscope focus knob towards you will move the objectives closer towards the specimen (= focus into the specimen). Turning the focus knob away from you will lower the objective, i.e., move it away from the specimen. For z-stack acquisition, the START is in the lower position of the objective. As the stack is being acquired, the objective is raised each step (moving against gravity), until the END position is reached. Turning the microscope focus knob towards you will move the objectives closer towards the specimen (= focus into the specimen). Turning the focus knob away from you will lower the objective, i.e., move it away from the specimen. For z-stack acquisition, the START is in the lower position of the objective. As the stack is being acquired, the objective is raised each step (moving against gravity), until the END position is reached.

**To set z-stack start and end position:**

1. Start Live View ("Focus x2" or "Focus x4" buttons in the software)
2. Focus out (lower the objective) until the object of interest is no longer visible in the Live View window.
3. Click the "START SET" button in the confocal software to set the START position.
4. Focus into the specimen (raise the objectives) past the object of interest.
5. Click the "END SET" button in the confocal software to set the END position.
6. In the confocal software, set the desired z-step size.

## Confocal Aperture (Pinhole) Size Calculation

When using dyes from the dye database, the Confocal Aperture size can be set automatically equal to1Airy unit, for good z-resolution. When the detection wavelengths are manually assigned in the "Light Path" panel, the software no longer calculates the Confocal Aperture size. In such case, use this formula:

$$PinholeSize\ (1Airy) = \frac{4.6 \times M \times \lambda}{NA}$$

where M = objective magnification
$\lambda$ = wavelength of the detected signal (in micrometers)
NA = numerical aperture of the objective

Axial resolution may be increased by closing the confocal aperture to a smaller value (to about 0.6x Airy), but this causes significant loss of signal intensity.

## Using objectives with coverslip thickness correction

The dry 40x/0.9 and water immersion 60x/1.2 objectives provide high resolution and good signal intensity only if coverslip of correct thickness is used. These objectives are equipped with an adjustment collar to match the actual coverslip thickness. If the adjustment collar is not properly set, resolution and signal intensity are degraded.

**Use coverslips # 1.5 for your specimens.** Their thickness is typically in the 0.16-0.19 mm range, and will vary from batch to batch, and also between individual coverslips. For high-resolution imaging, the objective correction collar must be set to minimize spherical aberration. This setting depends on the coverslip thickness and also on the specimen itself (refractive index, distance from the coverslip). The best way to set the correction collar is during visual observation, where the objective is focused at a bright speckle in the specimen and while repeatedly focusing up and down, the correction collar is turned to achieve a symmetry of the out of focus pattern of the point object (symmetrical point spread function).

For less critical imaging, the correction collar is simply set to the exact thickness of the coverslip. Since the standard coverslips vary greatly in their thickness between batches, each individual coverslip must be measured either using calipers before mounting, or using the confocal microscope on the prepared slide, as described below:

1. Use either a 40x dry objective or the 60x water immersion objective.
2. In transmitted light, focus on the specimen side closest to the coverslip.
3. Choose reflection mode on the confocal microscope with a small pinhole diameter (80 µm), use the neutral 20/80 beamsplitter in the illumination path and set the detector bandwidth to 10 nm, centered on the emission line of the laser (for example 538-548nm detection for the 543nm line of the HeNe-laser).
4. In the Focus4x mode, start focusing out of the specimen and watch the live image. You should get a bright reflection when focused at the interface of the coverslip and the specimen mounting medium. Adjust detector High Voltage (HV) as needed to get bright, non-saturated signal. Set the current z-position as 0 and continue to focus out of the specimen. You will again see a bright reflection when focused on the outer surface of the coverslip. Adjust the detector HV as needed. Please note that there may be several reflectance peaks at the outer surface of the coverslip, find the focus position with the brightest signal. Note the current focus position (= z difference between the two intensity peaks; example: 157 µm).
5. Calculate correction for the refractive index mismatch:

$$\mathbf{RC = \eta_2 / \eta_1}$$

where $\eta_2$ is the refraction index of the coverslip glass (1.525) and $\eta_1$ is the refraction index of the media in between the lens and the coverslip. $\eta_1$=1.0 for air, 1.33 for water. Therefore, the correction value for water immersion objectives should be about 1.525/1.33 = 1.146 Please note that this correction factor is accurate only for low-NA objectives.

5. Multiply the measured z-difference value by the correction factor (RC) to obtain the exact coverslip thickness (example: 157 µm z-difference x 1.146 = 180 µm = 0.18 mm).
6. Adjust the correction collar on the objective to the coverslip thickness.

## Saving Image Files

- By default, your image files are saved in **D:\FV10-ASW\users\yourusername\Image**. Create sub-folders there as needed.
- There are two native file formats, ".oib" and ".oif" It is recommended to use the ".oif" file format, since it saves 16-bit TIFF files that can be imported into other imaging programs for further analysis and processing.
- When finished, copy your files onto your media (CD ,DVD, USB drive). Remember to **copy both the ".oif" file and the associated sub-folder containing the actual images**. 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 without errors, erase the files from the computer.
- Old image files will be purged periodically to free disk space.

## Storing your data

- 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, e.g one in the lab, the other one at home.
- 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.

## Viewing and processing the image files

The native file formats (".oif" and ".oib") can be opened with the Olympus confocal software (there is a dedicated computer with this software in MIC's computer room) or with the free Olympus confocal image viewer that can be downloaded from our website (look in the "Instruments" page in the "Olympus confocal" section).

If the data were saved in the ".oif" format, the individual TIFF files for each detection channel and z or time can be imported into other image analysis programs, e.g., the freeware ImageJ software (http://rsb.info.nih.gov/ij/): "File"-"Import"-"Image Sequence".
Direct reading of these confocal datasets and other proprietary formats is also possible via the "LOCI" plugin for ImageJ (see http://www.loci.wisc.edu/ome/formats.html).

The off-line data processing computer also has the VOXX volume rendering software installed. It can load TIFF image stacks (e.g. those created by ImageJ from individual channels and z-sections) and create rotating 3-D or 4-D views (XYZ, or XYZ and Time).