

Fluorescence Imaging with Olympus IX81 Microscope

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Each new user must be trained **by the MIC staff**. Second-hand learning from another user is not sufficient to gain user status.

Even though no lasers are used for wide-field fluorescence imaging, the microscope is a part of the laser scanning confocal system and all users **MUST** take the online Laser Safety course, located at <https://ehsdtraining.tamu.edu>, before their first training session.

Standard operating procedure (SOP) and a copy of Texas Administrative Code §289.301 (Registration and Radiation Safety Requirements for Lasers) are located in the confocal microscope room.

The last user must be able to properly shut down the entire system, including the lasers if they were left on by the previous user.

Mercury Lamp Precautions

- The lamp emits strong UV and visible radiation. Do not look into the source or disassemble the lamp housing.
- **Do not turn the lamp on if the lamp usage counter reached its expected lifetime (300hr)!**
- 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.

Acknowledgment policies

MIC guidelines mandate that the use of the 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.

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

Biosafety requirements and rules for work in the MIC

Selected rooms in the Microscopy and Imaging Center (MIC) have been approved as BL-1 space. In order to be able to bring the active BL-1 material to the MIC, the MIC facility and room number **MUST** be listed in the investigator’s IBC permit, in Section F, Agent use and Storage Locations. The investigator is required to send a copy of the IBC permit listing the relevant MIC lab and the BL1 organisms, to MIC biosafety officer (vitha@tamu.edu) **BEFORE** bringing the BL-1 samples. The MIC is neither equipped nor allowed to deal with samples that are Biosafety level 2 (BL-2) or higher. If such samples need to be examined in the MIC, they must be rendered inactive prior to their transport to MIC. The investigator should first contact the University Biosafety Committee (IBC) and have an approved operation procedure for sample inactivation and containment. The investigator should consult with MIC to make sure that the procedure is compatible with microscopy imaging.

F. Agent use and storage locations.

Location ID	Campus	Building Number	Room Number	Room Use	Current Bio-safety Level	Shared Lab?	Other PIs
1	Texas A&M	1530	1116	Multiphoton microscopy	BSL-1	Yes	*
2	Texas A&M	1530	1117	Cell and tissue culture	BSL-1	Yes	*
3	Texas A&M	1530	1118	Confocal microscopy	BSL-1	Yes	*
4	Texas A&M	1530	1121	laboratory	BSL-1	Yes	*
5	Texas A&M	1530	1120	Micropsopy room	BSL-1	Yes	*

* The list of other PIs is maintained by the MIC

All users of rooms listed in table F must follow the rules. This applies even to those users that do not work with samples requiring IBC permit:

I) For users that DO NOT work with BL-1 agents:

- Closed toe shoes are required in the confocal microscope room. Upon exiting the microscope room, users are required to wash hands. The sink in the Bioprep lab (Rm. 1121) or in the Culture room (Rm. 1117) may be used.

II) For users working with active BL1-agents:

- The MIC facility and room number **MUST** be listed in the investigator's IBC permit, in *Section F, Agent use and Storage Locations*; The PI is required to send a copy of the IBC permit, listing the relevant MIC lab and the BL-1 organisms, to acurington@mic.tamu.edu **BEFORE** the BL-1 samples can be brought to the MIC. Without the MIC facility listed in the PI’s permit, no BL-1 work is allowed in the MIC.
- Samples being brought to the MIC must be contained, in accordance with the operating procedure in investigator’s IBC permit, to prevent spills during transport

- Closed toe shoes must be worn
- The use of gloves should be restricted to only handling the sample to avoid contaminating general work area. Touching the microscope or the control computer keyboard and mouse with gloved hands should be avoided. Gloves may not be worn outside of the rooms listed in table F.
- The user must ensure proper disposal of the samples - taking all samples as well as contaminated microscope slides, etc..., back to his/her laboratory for disposal. Samples may not be put in regular trash in the MIC.
- Any spills and contaminations must be cleaned up, decontaminated by the user and the emergency contact person notified.
- At the end of the work session, the work area, including the microscope stage must be wiped clean using a disinfectant (70% ethanol or disinfecting wipes). Detailed instructions will be given by MIC staff as needed to ensure proper disinfection, while preventing damage to the instrument.
- Upon exiting the BL-1 work area, users must wash hands. The sink in the Bioprep lab (Rm. 1121) or in the Culture room (Rm. 1117) may be used.

Biological Spill Response: BL1 Laboratory

The following procedures are provided as a guideline for biohazard spill clean up in a BL1 laboratory. Although the biological material in a BL1 level spill should not be a significant health hazard, you have the obligation to minimize the release of recombinant organisms and biohazardous material from the laboratory.

In the event of a spill:

- If a biohazardous material spills on you, remove any contaminated clothing and wash any exposed body parts.
- If a biohazardous material gets in your eyes, flush at the nearest eyewash station.
- If the spill area is large or in a common use area, mark, label, or otherwise denote the area so others may avoid it.
- Using materials from your spill kit:
 - Put on gloves, lab coat, and eye protection.
 - Cover the spill with absorbent material.
 - Pour disinfectant over the entire area. Allow the area to soak for 30 minutes.
- If warranted, contact the principal investigator, assess the magnitude of the spill, and formulate further plans of action.
- Safely pick up any broken glass with forceps or sweep into a dustpan, and dispose the residue into a broken glass/sharps container.
- Place spill materials into an autoclave bag.
- Make sure area is cleaned and disinfected thoroughly.
- Soak any contaminated clothes and shoes in a tray with disinfectant.
- If the spill is greater than 25 ml or contains recombinant DNA, report the spill to the Office of Research Compliance and Biosafety at 979.862.4549.



<http://rcb.tamu.edu>

STARTUP PROCEDURE

1. Fill out the log sheet, **including the account number and your full name or last name.**
2. Turn on the following components (the order is important!):
 - Mercury Lamp Power Supply. **If the time counter exceeds 300 hours, do not turn it on,** ask MIC Staff to replace the Hg lamp)
 - Microscope Control Box,
 - Prior Optiscan stage controller
 - Camera – **Must be turned on before starting the acquisition software.**
 - Computer
3. Log in the computer: User = Fluoview Password =
4. Open the software: “Micromanager”. When prompted for the name of the configuration file, choose the generic “MMConfig_MIC_Prime.cfg” or select your own config file, located in the MicroManager installation directory "C:/Program Files/Micro-Manager-1.4/"

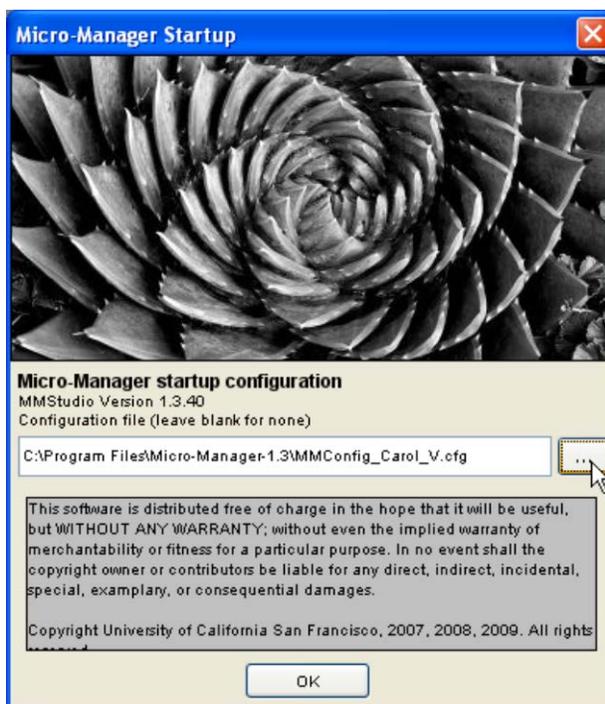


Figure 1. Loading a configuration file

The software now tries to take control of the microscope and other hardware. If you get error messages, try re-loading the hardware configuration (Tools - Load Hardware Configuration). If the Photometrics Prime camera was not detected, you will have to turn the camera on, wait ~30 s and restart the software.

You can change/modify the configuration file as needed and save it with a different name.

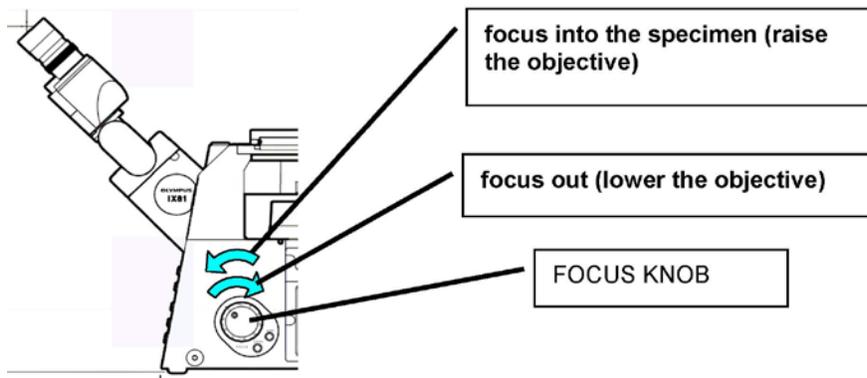
Using the Olympus IX81 microscope

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.

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.

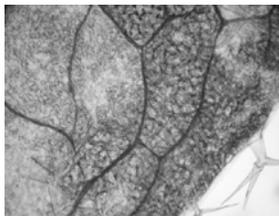


Setting up Köhler illumination

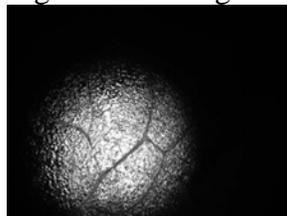
Köhler illumination produces an even field of illumination and does not show dust and dirt that accumulated on optical surfaces in the illumination path.

1. Place specimen on the stage, turn on the transmitted light (Trans lamp button in the software).
2. Focus the specimen (Fig. 1A). **From now on, do not touch the stage focus knob.**
3. Completely open the Condenser Aperture (on the condenser) and completely close the Field Aperture (on the arm on top of the plastic enclosure) (Fig. 1B).
4. Move the condenser up or down to focus the edges of the field aperture. The specimen and the Field Aperture must be in focus simultaneously (Fig. 1C).
5. If the Field Aperture is off center, use the two adjusting screws to center the aperture (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.
7. Use the Condenser Aperture to change contrast in the transmitted light image – closing the Condenser aperture will provide more contrast but less resolution.

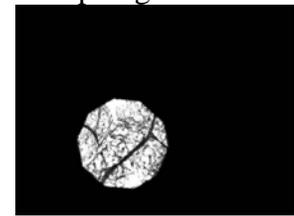
Figure 1: Köhler illumination - Focusing and centering the condenser Field Diaphragm



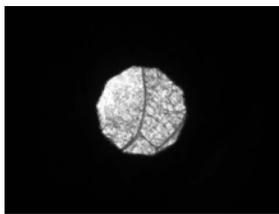
A: Focus the specimen



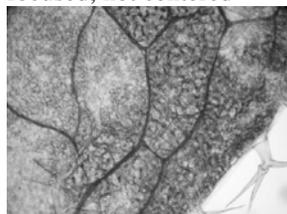
B: Field Diaphragm closed down, not focused, not centered



C: Field Diaphragm focused, not centered



D: Field Diaphragm focused and centered



E: Field Diaphragm opened just enough to disappear from view

Differential Interference Contrast (DIC, a.k.a. Nomarski) observations

Set up Kohler illumination first.

Make sure the polarizer in the condenser is engaged

Check that the Filter set selected in the software is DIC.

The slider with Nomarski prism below the objective needs to be in the optical path (pushed in). The “hockey stick” handle on the slider should be set according to the objective used: pulled out for dry objectives, pushed in for immersion objectives. (*Explanation: This handle controls the distance of the Nomarski prism from the flange of the objective. Dry and immersion objectives have their back focal plane at a different position, The Nomarski prism must be close to the BFP for best performance.*)

If the objectives are switched using the software’s pre-set group, the correct Nomarski prism is automatically engaged (and the objective turret retracts before turning to prevent damage or contamination of the objectives).

If you use the buttons on the keypad and on the microscope to switch objectives, you have to manually switch to the correct Nomarski prism in the condenser turret.: Each objective requires a different condenser prism for DIC to work. See table 1 below. Use the control keypad to turn the condenser turret (two buttons on the keypad, labeled “GFP” and “Cy3”).

Table 1: Condenser turret positions for DIC observation with individual objectives

Objective	Condenser turret position	“hockey stick” handle position on the DIC objective prism
UPLSAPO 10x/0.4 dry	5	OUT
UPLSAPO 20x/0.7 dry	5	OUT
UPLSAPO 40x/0.9 dry	2	OUT
UPLSAPO 60x/1.2 water imm	4	IN
UPLSAPO 100x/1.4 oil imm	6	IN
UPLSAPO 20x/0.85 oil imm.	5	IN

Using the 20x and 40x dry objectives- how to keep them clean:

If the slide is clean and does not have any immersion oil, you may switch to the 20x or 40x dry objective. To do so, either switch the objectives via software, or press the "ESC" button on the microscope (green button behind the focusing wheel) - this retracts the objective turret to prevent damage or contamination of the objectives. Then use the control keypad to switch to the desired objective and push the "Esc" button again to return to the focus position. You should need only minimal re-focusing (1/4 or 1/2 turn of the focus wheel).

Beware that if your slide already has immersion oil on it, switching to the 20x or 40x objectives will get oil on these dry lenses and will require their careful cleaning by the MIC staff. If this happens, please let the MIC staff know and do not try to clean the lenses yourself. The front lens elements are very delicate and prone to scratching, which would ruin the optics.

Using the oil immersion objectives:

- Remove the sample from the microscope
- Select the "100x oil" or "20x oil" objective in the software
- Apply a small drop of immersion oil on the objective
- Press the ESC button on the microscope to lower the objective
- Put the sample (slide) on the microscope stage in the same orientation as it was before.
- Press the "ESC" button to bring the objective into focus

CLEANING

- NEVER USE KIMWIPES OR OTHER TISSUE PAPER TO CLEAN OBJECTIVES. **USE ONLY LENS PAPER** (e.g. Fisher 11-996)
- 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.
- Wipe off any oil from the objective barrel.
- Thorough cleaning of the oil immersion objectives is performed by the MIC staff only.

Using the 60x/1.2 Water Immersion objective

- Remove the sample from the microscope. Make sure the coverslip on your slide is clean and dry, with absolutely no oil
- Select the 60x/1.2 water immersion objective in the software
- Set the adjustment collar on the objective to the thickness of the coverslip on your specimen (Coverslips #1.5 are approximately 0.17 mm thick)
- Apply a small drop of clean ultra-filtered water on the objective, using a plastic Pasteur pipette
- Press the “ESC” button on the microscope to lower the objective turret
- Place the sample on the microscope stage in the same orientation as it was before
- Press the “ESC” button to bring the objective into focus

CLEANING the 60x/1.2 Water Immersion Objective

- NEVER USE KIMWIPES OR OTHER TISSUE PAPER TO CLEAN OBJECTIVES. **USE ONLY LENS PAPER** (e.g. Fisher 11-996)
- Using clean lens paper gently blot off the water 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.
- Thorough cleaning of the oil immersion objectives is performed by the MIC staff only.

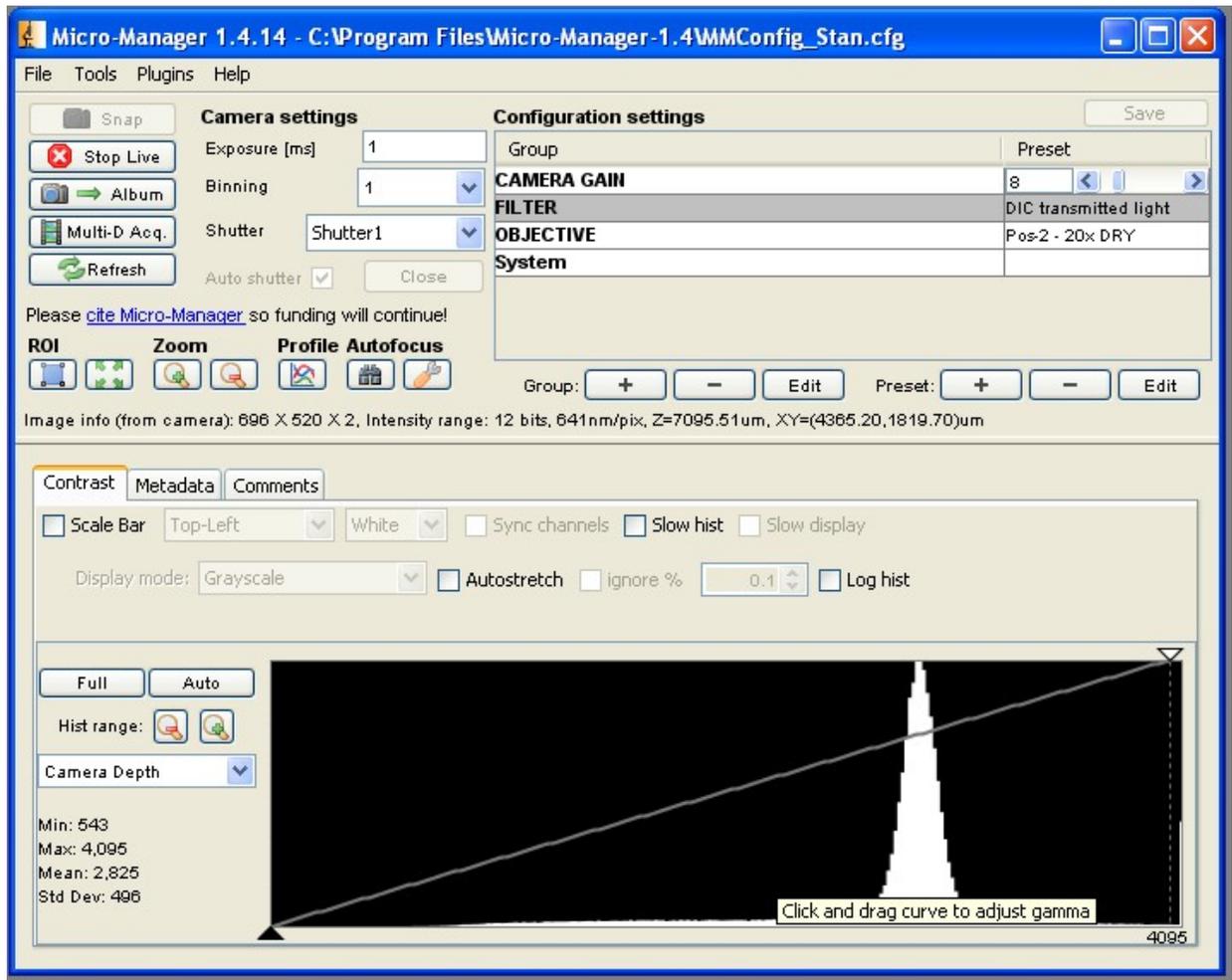
The importance of coverslip thickness for image quality

Use coverslip #1.5 (~0.17 mm thickness) for best image contrast and resolution. The microscope objectives work best with those coverslips. The 40x dry and 60x water immersion objectives have correction collars for coverslip thickness compensation, but they are hard to reach. It is best to leave them adjusted at 0.17 mm and use the correct coverslip.

Fluorescence microscopy

- Focus on the specimen using transmitted light (DIC), if possible
- Switch to the desired filter in the software (e.g., GFP)
- Switch from the camera port to eyepiece observation (top left button on the front of the microscope)
- Open the mechanical shutter below the objective turret. Illumination light can be attenuated using a lever on the mercury lamp housing
- Find and focus the specimen
- If you will not be acquiring images right away, close the mechanical shutter or switch back to DIC filter set in the software
- Beware that the Nomarski prism below the objective degrades the fluorescence image. Thus, if high resolution imaging is needed, pull the Nomarski prism out of the optical path

Camera imaging

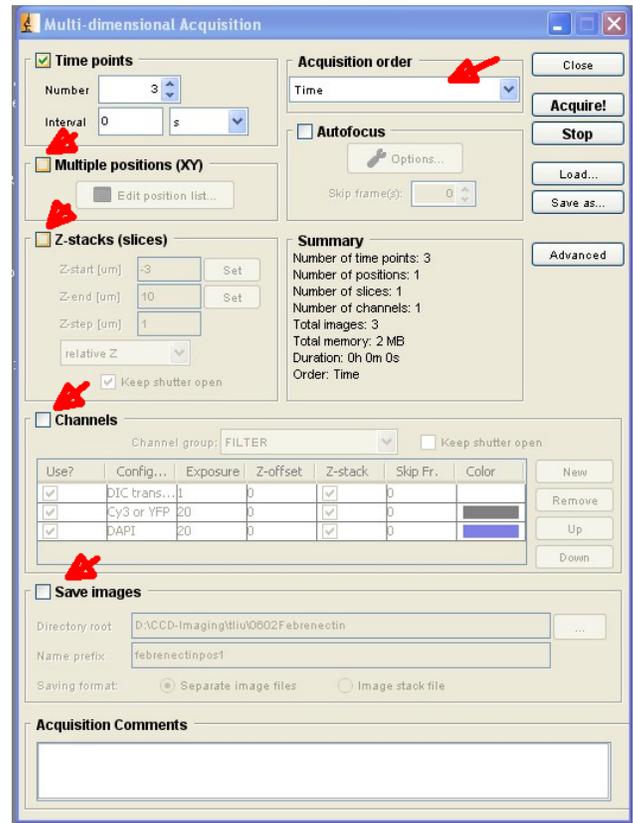


- Select the desired filter set in the FILTER menu.
- Select the desired objective in the OBJECTIVE menu. Take care not to get oil on dry objectives or on the water immersion 60x objective.
- Run "Live View" and set the Exposure to get a good image. You may need to adjust the histogram to display the image properly (Click "Auto", then tweak the Histogram Min and Max manually)
- You may also adjust the display gamma by pulling the intensity line up or down.
- Switch to the next filter and optimize exposure time as needed.
- To set up Multidimensional Acquisition, click the "Multi-D Acq" button

Multidimensional Acquisition

Set parameters as needed:

- Time Points
- Multiple Positions (XY): you can mark XY positions or define a matrix of XY coordinates
- Slices (Z) if doing z-stacks; set z-start, z-end and z-step (see Table 2 for optimal z-steps).
- Channels: set the filter, exposure time, z-offset (to correct for focus shift between different colors) and check whether to acquire the channel for every slice in a z-stack or just once. You can also set to skip a number of frames in a time lapse series.
- Assign a name to the experiments (directory and name prefix). Make sure "Save image files to acquisition directory" is checked.
- Select the acquisition order (e.g., Channels, slices, time points)
- Check the "Save Image" option, choose the destination folder and the file name.
- Usually, saving images as a single multi-image tiff is preferred.



XY Pixel size, Recommended Z-step, Sampling and Nyquist Criterion

The Photometrics Prime sCMOS camera has 2048 x 2048 pixels (6.5 μ m pixel size). To capture the image at full resolution, the smallest resolved detail in the primary image should be represented by at least two pixels (“**Nyquist criterion**”).

The default configuration file contains spatial calibration for each objective when used without additional magnification. As long as you switch the objectives using the button in the software, rather than using the keypad by the microscope, the pixel size is known to the software and saved with the acquired image. When additional magnification is introduced by engaging a 1.6x field lens (the handle on the microscope is pulled out), the spatial calibration is no longer valid.

If this is a problem and you will be imaging using the additional 1.6x magnification, you can change the spatial calibration (pixel size for each of the objectives) in your config file to the 1.6x field lens values. See table 2 below. You should then save your own copy of your configuration file.

Table 2 shows the pixel and resolution calculation. XY resolution is calculated as $D_{xy}=0.61 \cdot \lambda / NA$; Axial resolution is calculated as $D_z=1.4 \cdot \lambda \cdot \eta / (NA^2)$, where NA is the numerical aperture of the objective, λ is the wavelength of signal (here, assumed 500 nm, i.e., 0.5 μ m) and η is the refractive index of the immersion medium (air, 1.0; water, 1.33; oil, 1.515).

Table2. Pixel size for Photometrics Prime sCMOS camera on Olympus IX81 microscope. Resolution is calculated for 500 nm wavelength. **Green fill indicates that the XY pixel size satisfies the Nyquist criterion.** All other conditions result in **undersampled images**

Objective	NA	Imm. Medium RI η	Tube Lens	pixel size [μ m]	Calc. D_{xy} [μ m]	Calc. D_z [μ m]	Suggest. z-step $D_z/2.3$ [μ m]
10x/0.4 dry	0.4	1.00	1x	0.6470	0.763	4.38	1.90
10x/0.4 dry	0.4	1.00	1.6x	0.4034	0.763	4.38	1.90
20x/0.7 dry	0.7	1.00	1x	0.3227	0.436	1.43	0.62
20x/0.7 dry	0.7	1.00	1.6x	0.2019	0.436	1.43	0.62
40x/0.9 dry	0.9	1.00	1x	0.1596	0.339	0.86	0.38
40x/0.9 dry							
60x/1.2 WI	1.2	1.33	1x	0.1080	0.254	0.65	0.28
60x/1.2 WI							
20x/0.85 oil	0.85	1.515	1x	0.3205	0.359	1.47	0.64
20x/0.85 oil	0.85	1.515	1.6x	0.2004	0.359	1.47	0.64
100x/1.4 oil	1.4	1.515	1x	0.0654	0.218	0.54	0.24
60X/0.7 dry, LWD	0.7	1.00	1x	0.1121	0.436	1.43	0.62

Data management and conversion

Storing your data

- The imaging computer is not for long-term data storage. After copying your files to your storage medium and confirming they are OK, erase the files from the computer. Old data files will be purged periodically.
- 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

Recent versions of Micromanager allow 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 .

SHUTDOWN PROCEDURE

- Remove specimen from the stage
- Using lens paper only, gently blot excess oil from the oil immersion objective. Do not wipe the lens. NEVER USE KIMWIPES or other kinds of paper on the objective lens. The front lens is easily scratched. Thorough cleaning of the objectives is done by the MIC staff only.
- Switch to the lowest magnification objective (10x).
- Clean the work area.
- Turn off all control boxes and the computer, the Mercury lamp power supply is the last to be turned off.
- If you are the last user for the day, and if the lasers are still running, it is your responsibility to turn the lasers off!

Laser turn off procedure:

1. 405 nm diode lasers: turn the key to OFF, turn the power switch to OFF.
2. Red and green HeNe lasers – turn the knob to OFF **ONLY IF YOU ARE THE LAST USER FOR THE DAY**
3. Argon laser: **TURN OFF ONLY IF YOU ARE THE LAST USER FOR THE DAY. IF NOT, LEAVE THE ARGON LASER RUNNING.**
 - i. Turn the key to OFF position
 - ii. **WAIT!! The laser must cool down.** The fan to stop automatically when the laser unit has cooled down. It takes several minutes
 - iii. Set the power switch to OFF.

Sign off in the log book.

Turn the lights off; make sure the door is locked when you leave.