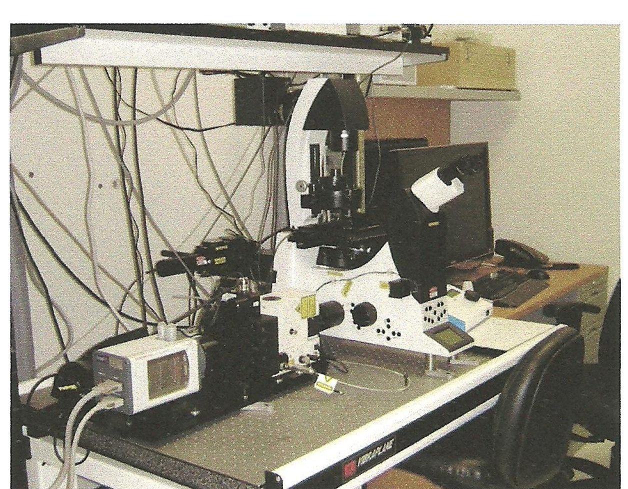
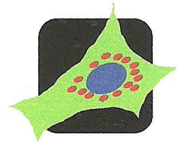
PLEASE LEAVE THIS DOCUMENT IN ROOM E-512

Quorum technologies WaveFX spinning disk confocal microscopy system
Manual and reference
Segal Cancer Centre/Lady Davis Institute room E-512

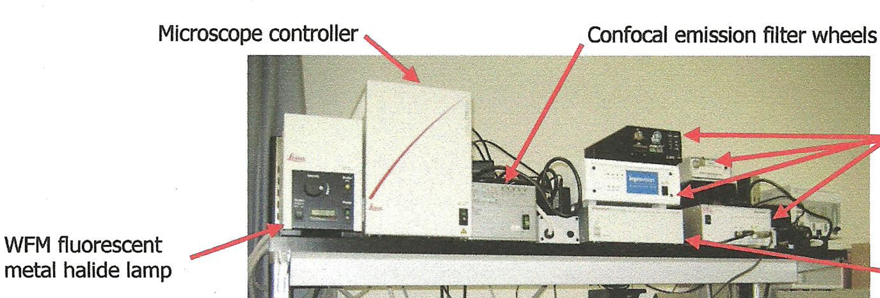




Equipment overview

Microscope controller

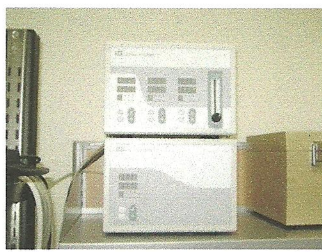
Confocal emission filter wheels controller



Motorized stage and FRAP components controls

WFM fluorescent metal halide lamp

Camera controller

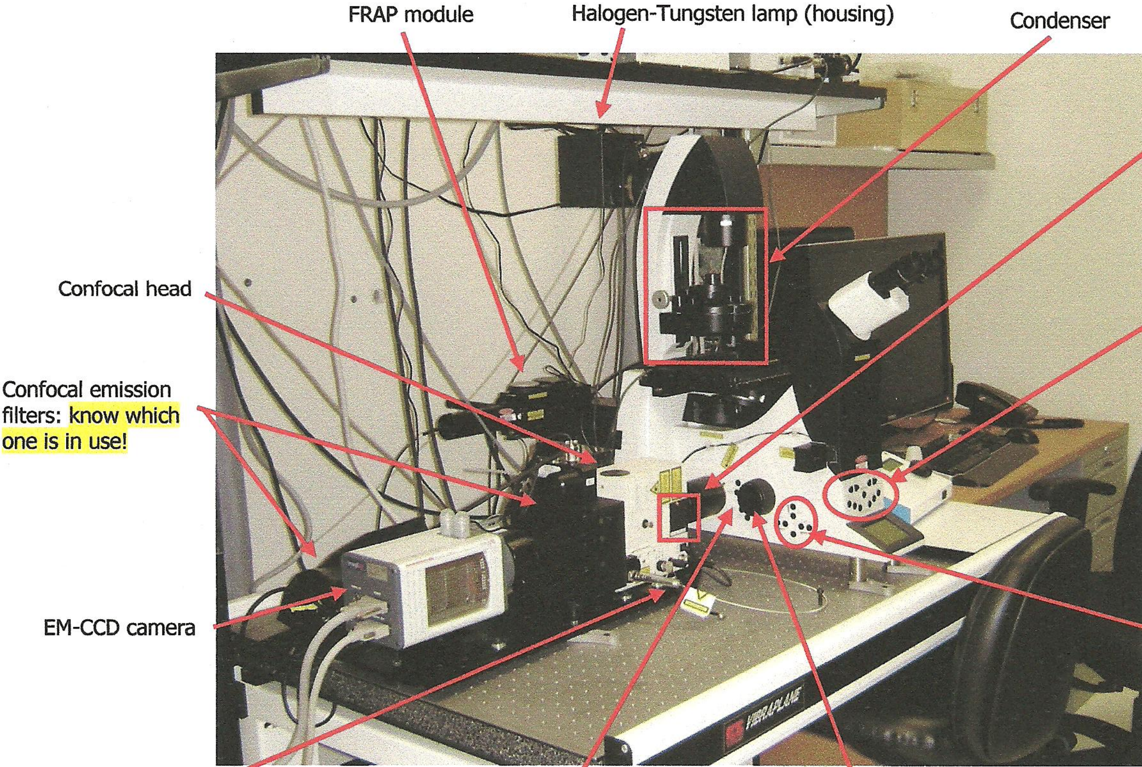


Environmental controls

FRAP module

Halogen-Tungsten lamp (housing)

Condenser



Dichroic: know which one is in use!

Confocal head

Controls for eye/camera toggle prism and WFM filters and shutter

Confocal emission filters: know which one is in use!

EM-CCD camera

TLM field aperture (AP), WFM field aperture (WFM), lamp intensity and TLM/WFM toggle.

Optical fiber FRAGILE!!

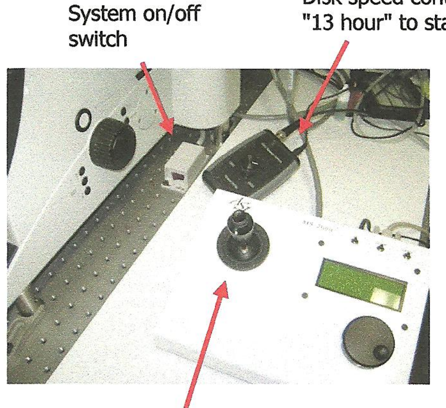
Controls for contrast modes (BF, PC, pol, DIC)

Fine/Coarse focusing knob

Confocal lasers, merge module and controls

System on/off switch

Disk speed control (set at "13 hour" to start)



Joystick for XY movement

Start up procedure

1	2	3
IF NEEDED, turn on the fluorescence lamp (Top shelf, to the left side).	Turn on the system's switch.	Turn on the laser control, then the required lasers:
3a: 405nm	**3b: 440nm**	**3c: 491nm**
IF NEEDED, turn the key such that the green light is on.	IF NEEDED, turn the key such that the green light is on.	IF NEEDED, turn the key such that the green light is on.
3d: 561nm	**3e: 642nm**	**4**
IF NEEDED, turn the key such that the green light is on.	IF NEEDED, switch to on.	-Turn on computer. -IF NEEDED, install sample holder.
5	**6**	**7**
IF NEEDED, install environmental control chamber (see instructions).	IF NEEDED, turn on FRAP system (see instructions).	-Ensure table is floating and levelled. -Start Volocity software. -Evaluate objective performance (qualitatively), and clean it IF necessary (see instructions).

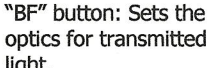
Shut down procedure

1	2	3
-In the Volocity acquisition software, close all shutters. -Exit Volocity. -Back up the data according to the established policies and erase them from the computer. - Log out of the account.	Turn off FRAP system (if it was used).	Remove sample holder or the environmental control chamber (see instructions)
4	**5**	**6**
Turn off ALL lasers.	Turn off the laser control.	Turn off the system's switch.
4	**5**	**6**
Switch off the fluorescence lamp.	-Lower the objective turret. -Wipe excess oil from surface of objective lens and from its sides. -Turn objective turret to an empty position. -IF the system was completely shut down, put the cover on the microscope.	

Operation of the Leica DMI 6000 microscope

This stand is highly motorized and hence very versatile too.

Left side functions

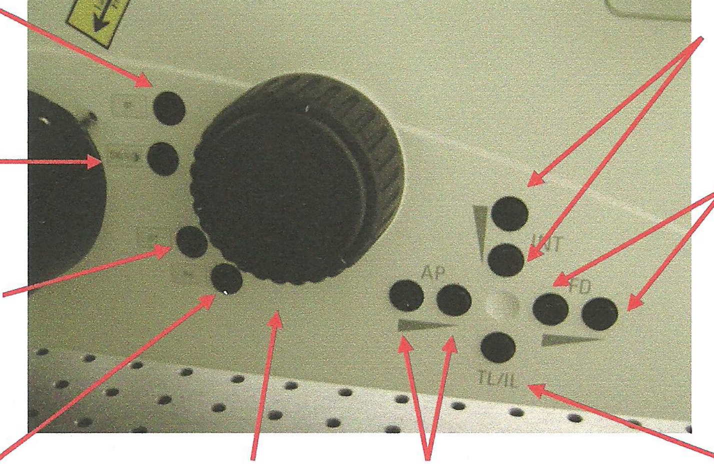


"BF" button: Sets the optics for transmitted light.

"CHG TL" button: Toggles through all the possible transmitted light modes (BF, PC, pol and DIC).

"ICT" button: Sets the optics for transmitted light differential interference contrast (DIC).

"PC" button: Sets the optics for transmitted light phase contrast (PC).



"INT" button: Adjustments for light intensity (either transmitted light or epifluorescence).

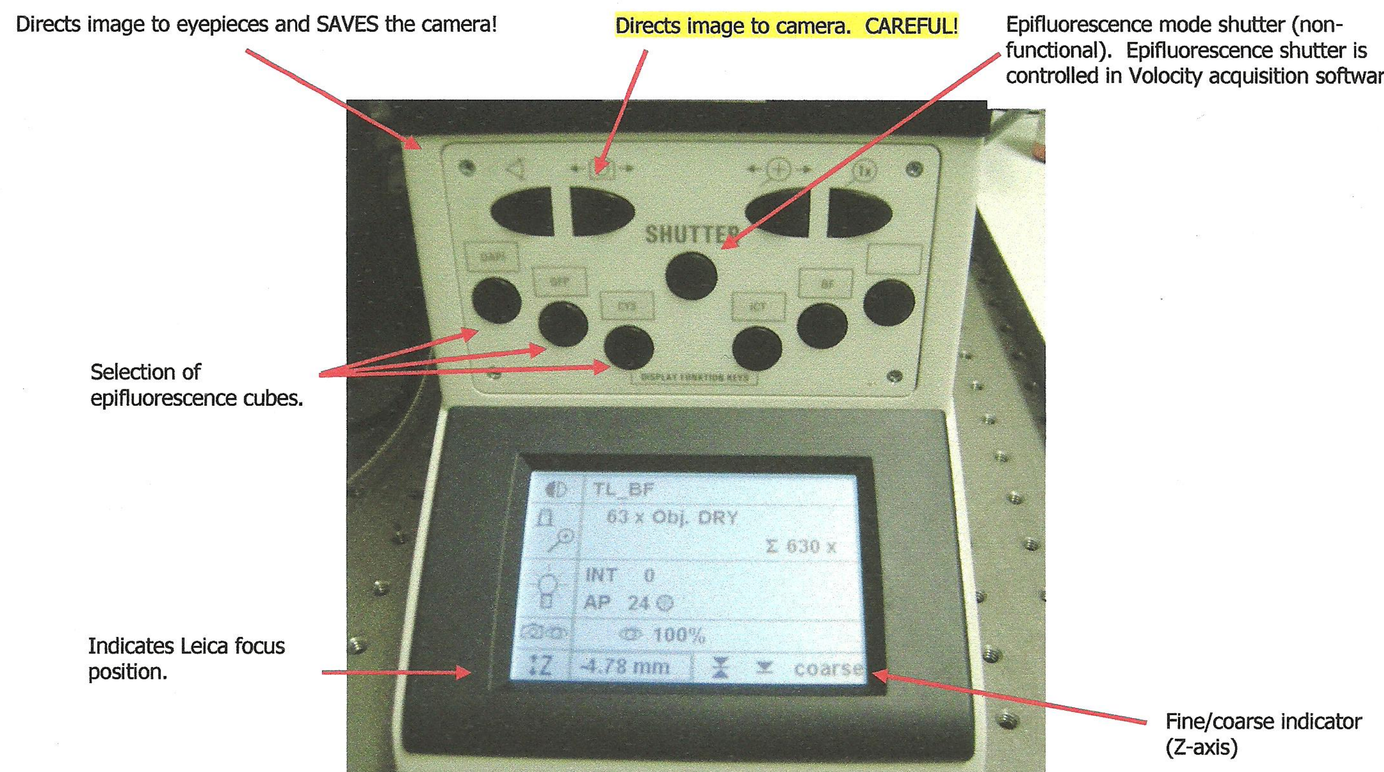
"FD" button: Adjustments for the field diaphragm in the epifluorescence mode.

Leica focus knob (toggles between fine and coarse, see right side functions).

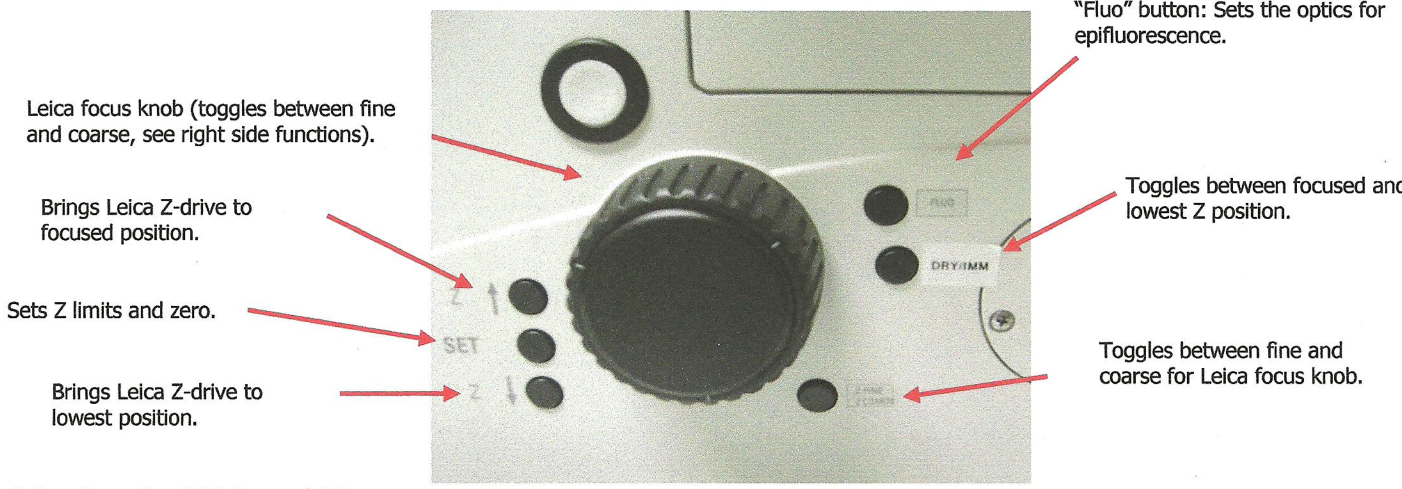
"AP" buttons: Adjustments for the field aperture diaphragm in transmitted

"TL/IL" button: Toggles between transmitted light modes and epifluorescence mode.

Front panel functions



Right panel functions



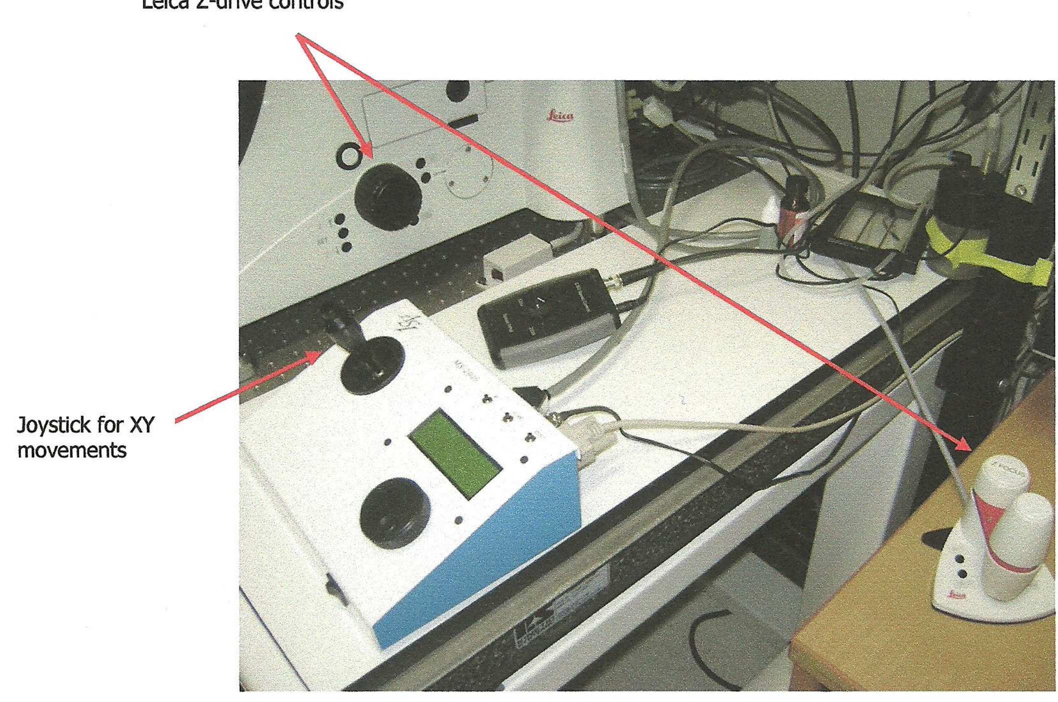
Moving in X,Y and Z

Moving in XY is achieved through the motorized ASI stage using the joystick (see below).

Moving in the Z axis is achieved by two options:

-The objective can be moved up and down using the Leica Z-drive. Both the Leica focus knob on the microscope stand and the SmartMove controller can be used (see below).

-The ASI stage can be moved up and down. This is done through the Volocity software (see later).



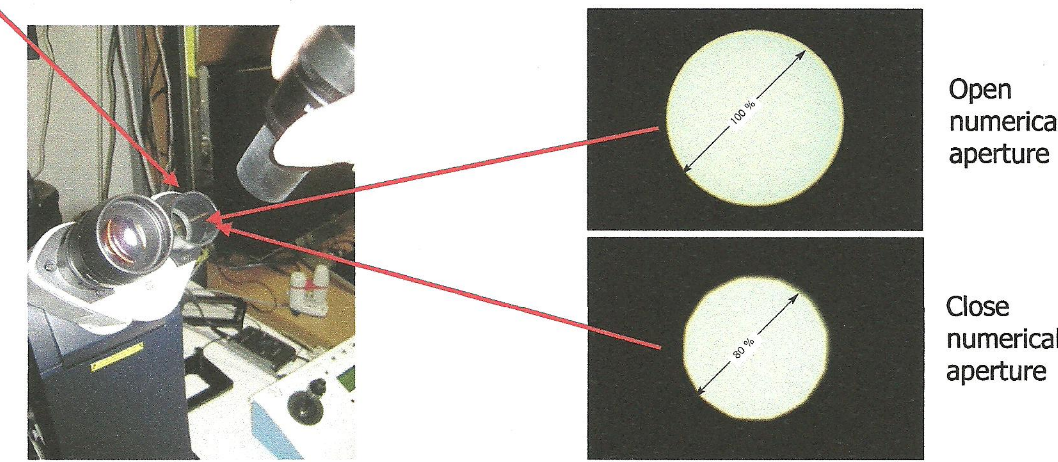
Condenser alignment (Köhler illumination)

Looking at a test slide using bright field can provide an assessment of the objective quality. Proper alignment of the condenser is crucial to evaluate the objective, but also for proper image formation in all transmitted light contrast modes (bright field, phase contrast, DIC etc). Condenser alignment needs to be done for each objective. There are two diaphragms to be adjusted: the field diaphragm (FD) and the field aperture (AP).

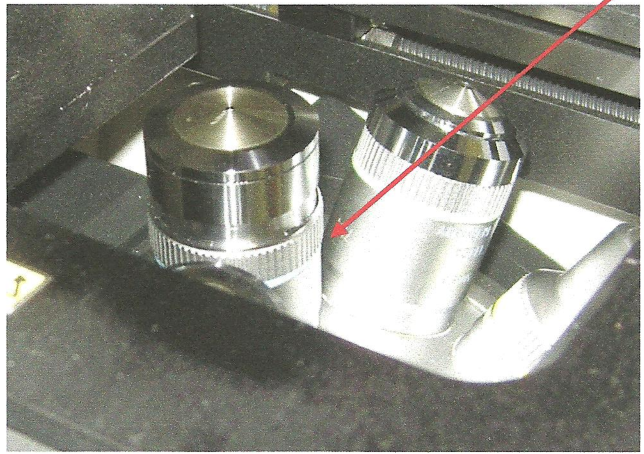
Checking the numerical aperture of the 40X and 63X objectives : IMPORTANT

The numerical aperture, hence the resolution power, of these two objectives is easily adjustable. For confocal imaging and for subsequent processing, analysis and deconvolution, it is absolutely essential to use these two objectives at the maximal opening.

The numerical aperture diameter can be observed by looking at the back focal plane when an eyepiece is removed:

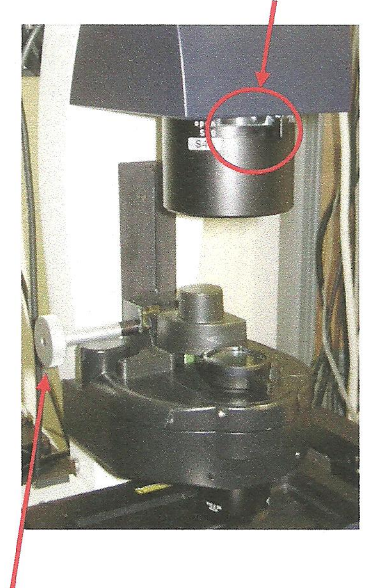


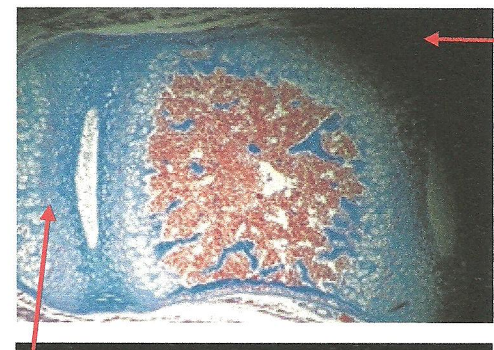
Focus on a test sample and remove an eyepiece. While looking down at the back focal plane, find the numerical aperture and open it at the maximal diameter by rotating the adjustment ring on the objective.



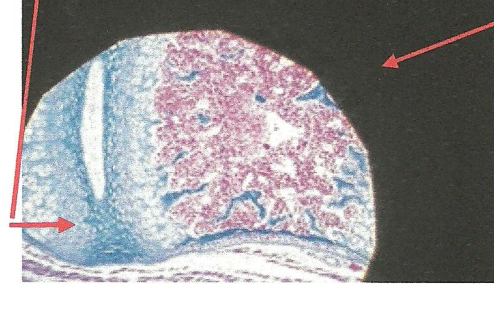
Adjustment of the Field diaphragm (FD)

If necessary, switch the light path to the oculars.
Switch on the halogen lamp and adjust light intensity for eye comfort.
Switch to the bright field transmitted light mode.
Using the 20X objective, focus on the test slide.
Close the field diaphragm (FD) until it (or a black shadow) appears in the field of view.





Side of FD
(out of focus)



Side of FD
(focused but not
centered)

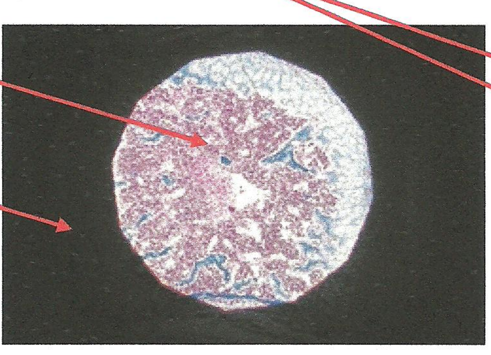
Focused
sample

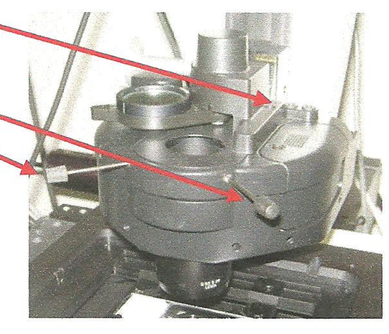
Focus the field diaphragm (i.e. adjust condenser's height) using the black knob on either sides of the pillar.

Center the field diaphragm using the two screws (stored at the back) at the front of the condenser.

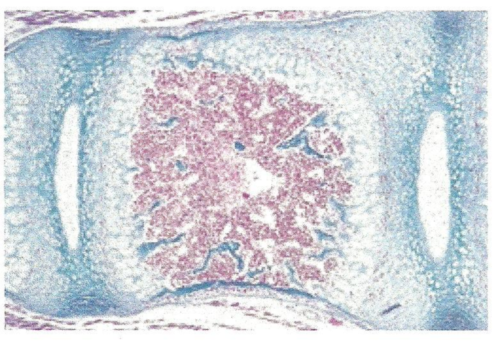
Focused sample

Focused and centered
field diaphragm





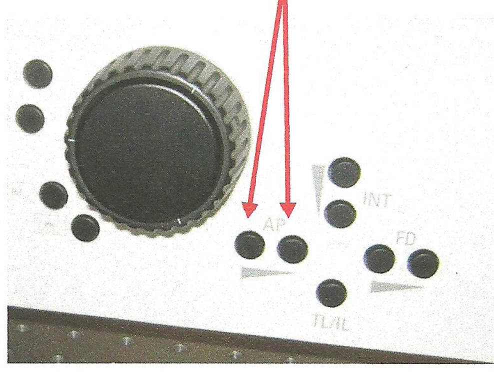
Open the field diaphragm just enough to have it out the field of view:

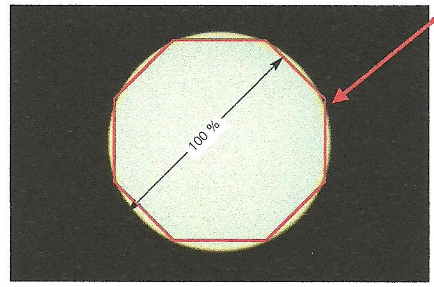


Adjustment of the field aperture (AP)

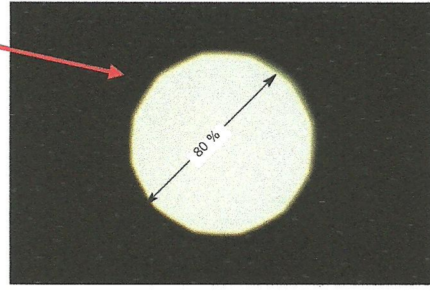
Remove an eyepiece and look for the aperture in the back focal plane of the objective.

Open or close the field aperture (on the left side of the microscope) to find the diameter that match the whole field of view (i.e. 100%).





Adjust the diameter of the field aperture to 80%.



Objectives and optics

IMPORTANT
ALL OBJECTIVES ARE DESIGNED TO WORK WITH GLASS COVERSLIP THICKNESS OF 0.17 MM (GRADE NO.1.5 GLASS). Assuming images were not binned, the following applies:
*In addition to bright field.

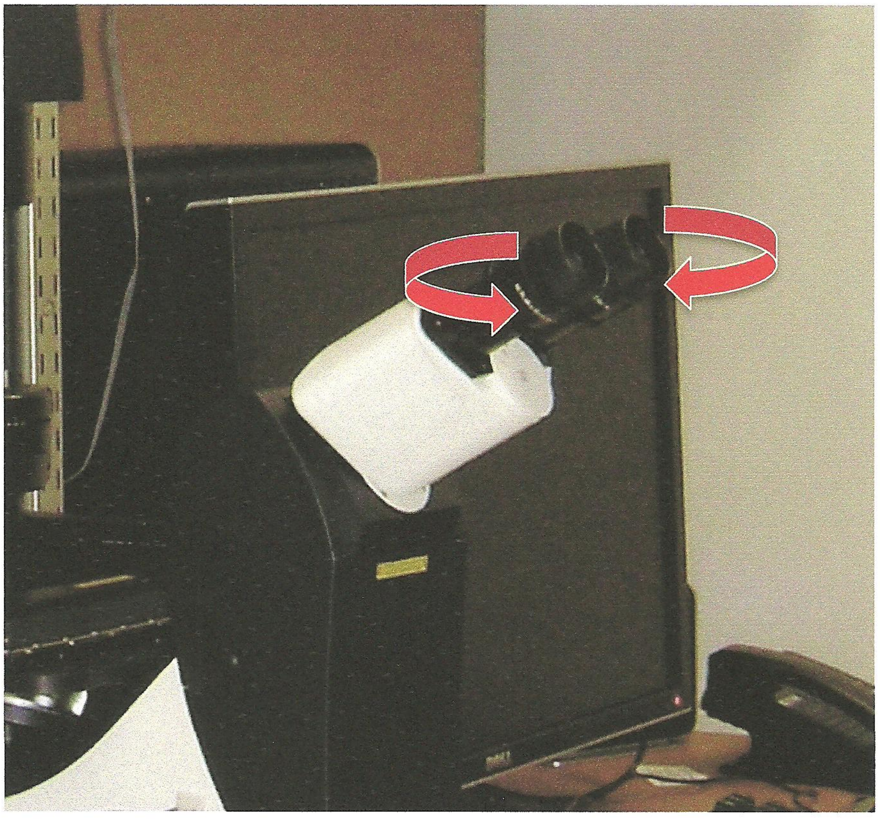
Objective	Contrast modes*	FA setting	um/pixel	pixel/um
10X/0.4 dry Ph1	Phase, Pol	12	0.9930	1.007
20X/0.7 dry	Pol	17	0.4937	2.0255
40X/1.25-0.75 OIL	Pol, DIC$	18#	0.2503	3.995
60X/1.4-0.6 OIL	Pol, DIC$	18#	0.1567	6.382

#Close FD completely.
$ Polarization and DIC are not possible through plastic.
NA 0.55 S28, WD: S70/40 condenser.

Parfocality adjustment

This adjustment will reduce photobleaching of the sample. It will render Z-stacks acquisition easier.

1. Using the eyepieces and transmitted light bright field, focus on the test slide.
2. Send the light to the left side port (the camera).
3. Using the fine focus knob, focus on an easily recognizable fine feature on the monitor.
4. Send the light to the eyepieces again. Look for the same feature down the eyepieces. It may be out of focus.
5. Refocus on the feature using the left eye and the left ocular.
6. Repeat the last step with the right side.



Polarization and differential interference contrast (DIC) microscopy

DIC illumination is a transmitted light microscope method. Like phase contrast, it provides more contrast than bright field microscopy alone, especially for thin, transparent samples such as cells.

When combining transmitted and fluorescence, DIC is preferable to phase contrast because the ring present in phase contrast objective is sufficient to block the little amount of light coming from fluorescent samples.

Properly setting up DIC illumination is highly dependent on proper alignment of the condenser.

Finding the focus using DIC, instead of fluorescence (e.g. DAPI), will significantly decrease fading and bleaching problem. With DIC, one can also evaluate the quality of the sample:

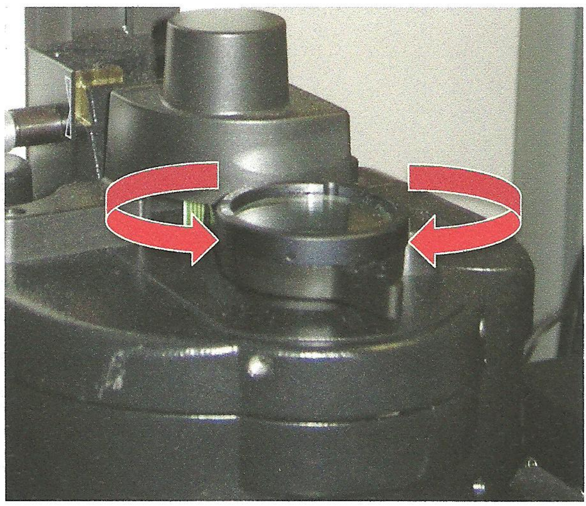
- cell confluency
- cell debris vs healthy cells
- Bacterial contamination

Light source
Polarizer
Condenser
Prism
Sample
Objective
Prism
Analyzer
Image

The elements required to do polarization and DIC microscopy are motorized. They are set in the light path by using the "CHG TL" button on the left side of the stand.

Crossing the polarizers: IMPORTANT

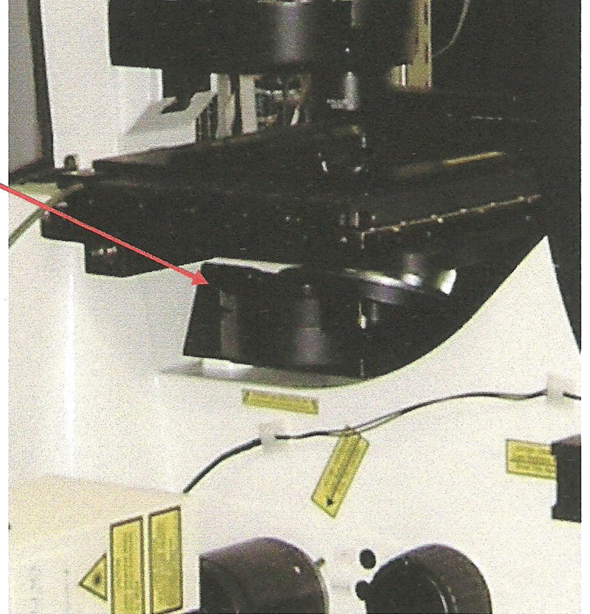
In properly set polarization microscopy, the background should be as dark as possible. This is achieved by rotating the polarizer. After focusing on a test slide, rotate the polarizer to obtain the darkest possible background on the test sample.



Properly set polarization is essential to perform DIC microscopy. It's also useful to detect the presence of crystals in the immersion oil as they show up as large unfocused bright spots freely swimming in the field of view.

Fine tuning the DIC optics

The angle of the DIC objective prism can be adjusted to improve the quality of the DIC image. While observing a test slide, rotate the dial to find a good DIC effects.

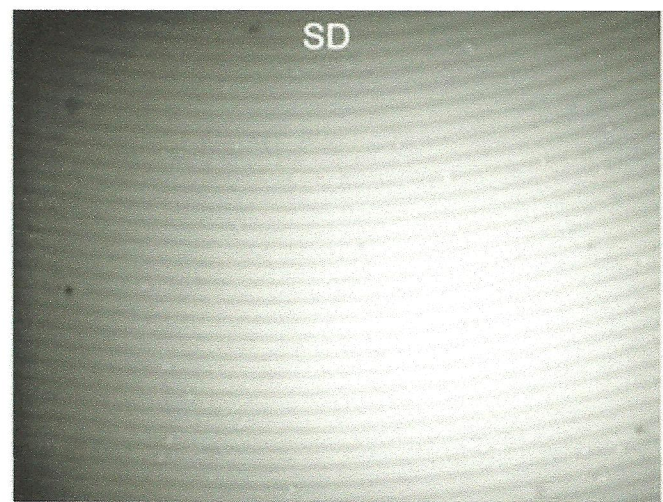


Relative to bright field, a good DIC image will present a dark background, high contrast of intracellular structures and a 3-dimensional perspective (when focusing up and down). If the DIC image doesn't form properly, ask the person in charge of the microscope to help you further.

Control of confocal disk rotation of speed and scan pattern artefact

The confocal spinning disk can rotate between 1200 and 2800 rpm. Because this rotation is not necessarily synchronized with the camera exposure time, there is often a scan pattern artefact. This is most commonly observed in bright field imaging at fast acquisition rates (e.g. 50 ms exposure). Increasing the exposure time greatly improves this situation. However if fast acquisition rates are essential, the artefact can be reduced by modifying the speed of the disk rotation.

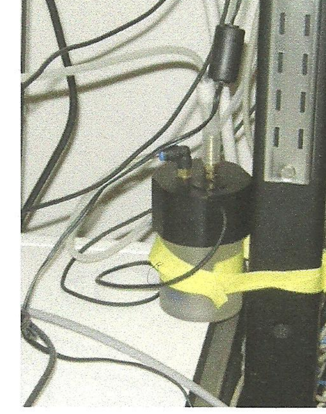
Procedure:
1. Determine the required camera exposure for the channel of interest (confocal, transmitted light etc).
2. Switch to bright field mode.
3. Modify the rotation speed and find the best setting.



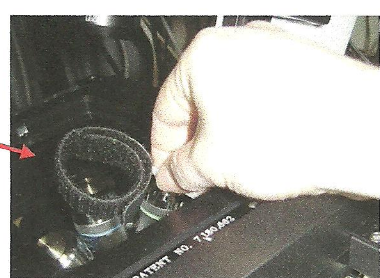
CO_2 stage incubator

Put high quality water in the humidifier and close it. Ensure it stands up right at all time.

Turn on CO_2 tank, ensures the gas is bubbling in the humidifier (left valve at 4-5psi).

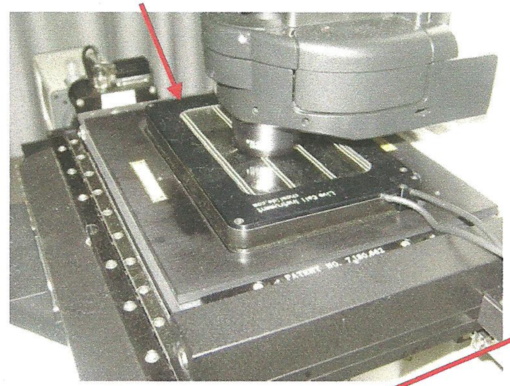


Install the objective heater if using the oil objective.



Install the main body and the appropriate insert.

Install the cover.



Turn on LCI chamber controller and the gas mixer and warm up (several hours to O/N). For this warming up period, one can install a dummy sample (e.g. 35mm glass bottom dish with a mark made with a black felt pen) containing water, and the chamber

cover. Use the black mark to focus and adjust condenser.

After the warm up period, check the temperature inside (using a probe thermometer), and adjust temperature if necessary.
Adjust temperature of humidifier if water condenses in the chamber.

ATTENTION!

1. Extreme precautions are to be taken when the stage incubator is installed on the microscope as the risk of damaging an objective is high.
2. Do not change objective once the objective heater is installed.
3. The humidifier must be STANDING at all time. Otherwise the risk of pushing water into the microscope stand, damaging both electronics and optics, is high.

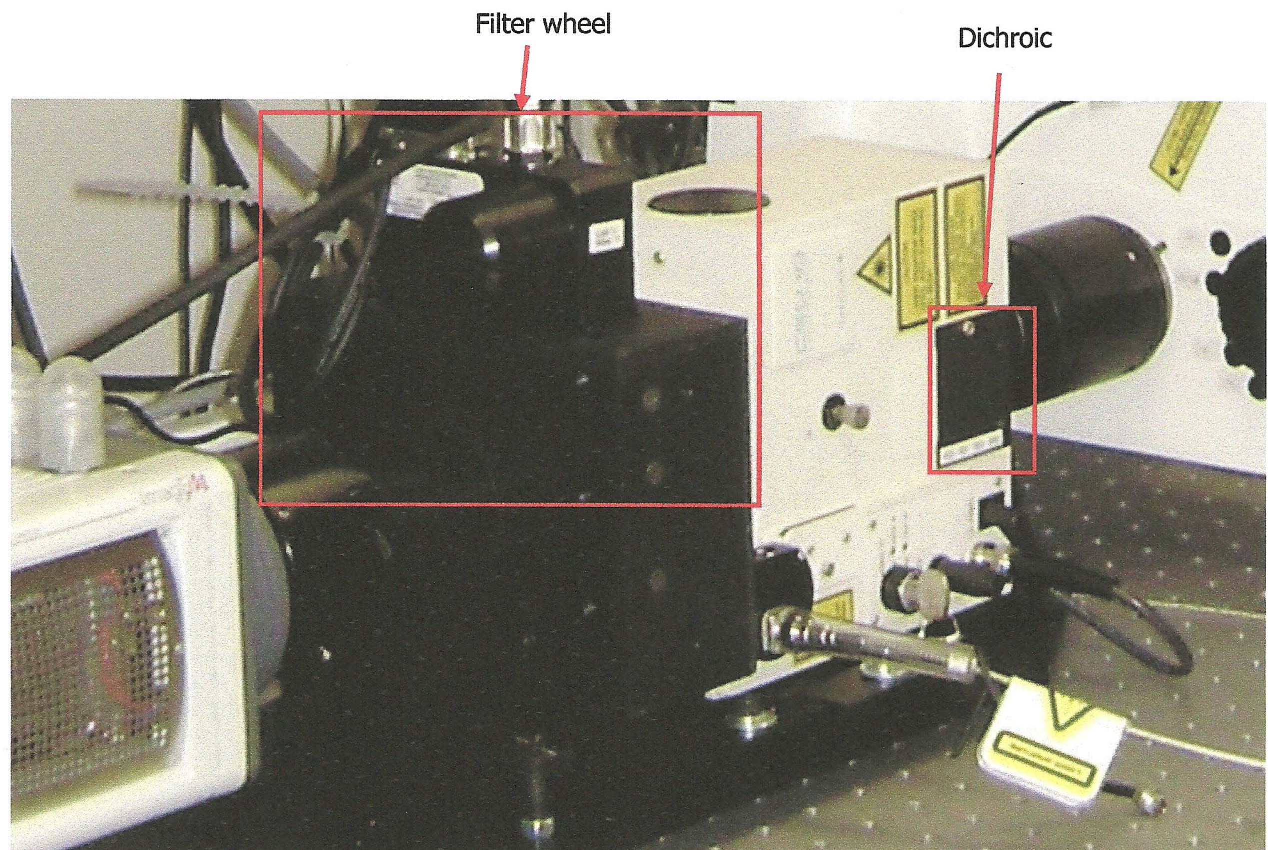
Dichroics and emission filter wheels for confocal imaging

There are two dichroics and two confocal emission filter wheels available on the confocal head of the system:

1. 405/491/561/640
-Usually installed on the system.
-To be used with Ludl filter wheel 1.
-Required to image DAPI.

2. 440/491/561/640
-Usually not installed on the system.
-To be used with Ludl filter wheel 2.
-Required to image CFP.

Make sure the right combination of dichroic and filter wheel is installed. Ask for assistance for the installation if necessary.



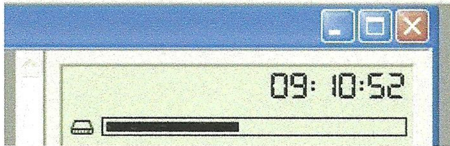
Improvision/PerkinElmer Volocity acquisition software

Make sure the system is already on.
Double click on the "Volocity" icon. This brings you to the Improvision License Server (ILS).
Provide your login and password and select the appropriate license:
3DMacquisition: to perform microscopy imaging.
RestoreClass: to perform deconvolution and measurements.
VisualClass: to perform 3D rendering and measurements.
Presentation: to use the free, limited edition of the software. Can be run without a license (useful to export data such tif and movie files).

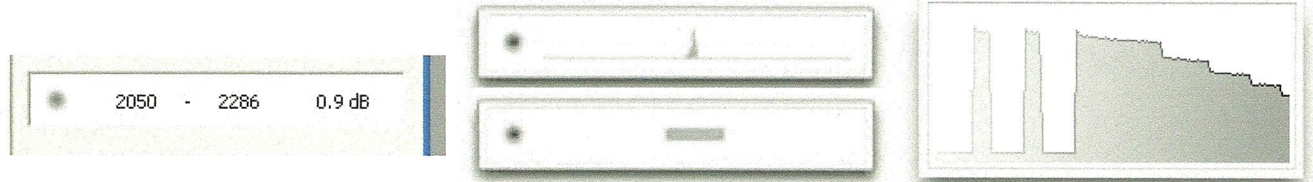
The software uses a database format to record both data (images per se) and metadata (e.g. objective used, laser power, filters etc). In Volocity, databases are called "Libraries".

Create a new library.
From Window menu, select "Show Video Preview"

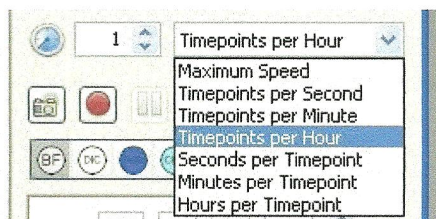
Video Preview menu



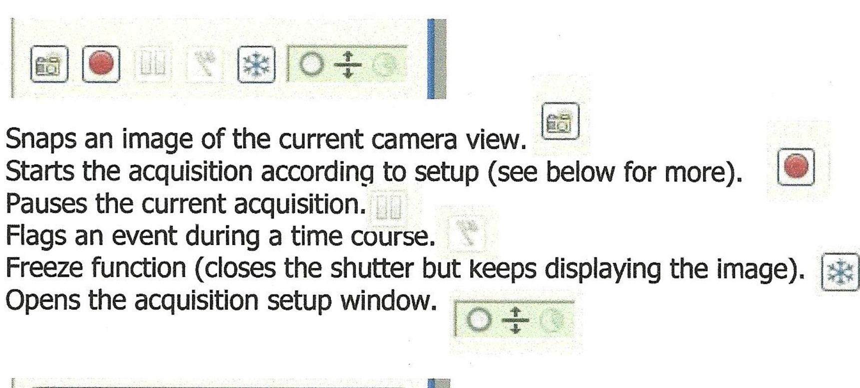
This green box shows time (real or elapsed) and hard disk space. Before starting a long time-lapse, ensure there will be enough space to save your data.



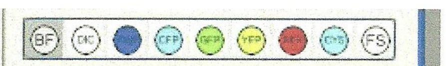
This white box displays pixel intensity information. The minimal and maximal pixel intensity values and signal-to-noise ratio (0.9 dB). One can also toggle to display other functions: histogram, bar graph and real-time maximum intensity display.



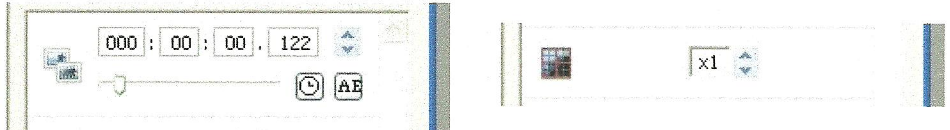
Control of the acquisition rate or frequency. Can also be accessed through the "Acquisition setup" (see below).



Snaps an image of the current camera view.
Starts the acquisition according to setup (see below for more).
Pauses the current acquisition.
Flags an event during a time course.
Freeze function (closes the shutter but keeps displaying the image).
Opens the acquisition setup window.



Light path selection. A maximum of nine light paths can be pre-programmed and every user of the system can modify any of them. Therefore, it is highly recommended to thoroughly check the light path to be used (see below for more details).

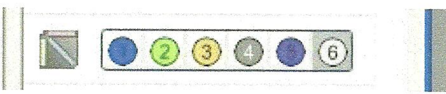


This slider controls camera exposure time. Camera binning should be x1 in most applications.

Auto Contrast should be activated for most fluorescence images. Camera EM gain is adjusted with the sensitivity slider.

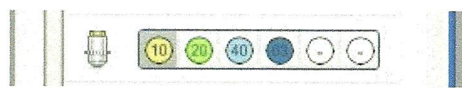
Set the camera read out speed to "High". Let the camera cool to -65°C before using it. Turn Photon Imaging off.

Make sure the right Leica contrast method is selected. All confocal light paths and bright field should be on the "TL BF" one. Polarization is "TL POL", differential interference contrast is "TL DIC", phase contrast is "TL PH". Wide field fluorescence is "Fluo". It is also possible to combine transmitted light (PC or DIC) with fluorescence using "TL PH/Fluo" or "TL DIC/Fluo" (for eye observations only).

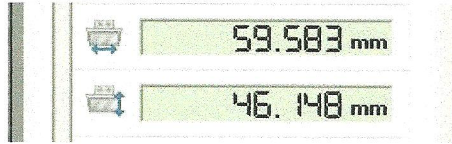


Make sure the Leica Fluorescent Turret is properly set. For confocal and bright light paths, it should be set to "Empty/Laser" (position 6). For polarization and DIC light paths, it should be set to "Analyzer" (position 4). The mosaic FRAP cube is in position 5 (see FRAP section). In wide field fluorescence microscopy, select the necessary fluorescence filter cube:
Position 1: A4 470/40nm (DAPI)
Position 2: GFP+ 525/50nm
Position 3: Y3+ 593nm 610/75nm

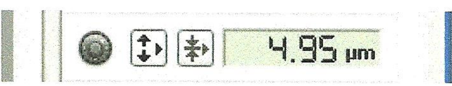
Indicator and control of the Leica focus drive (i.e. the objective turret going up and down). "Set Top" and "Set Bottom" will set the absolute Z positions of the Leica focus drive. "Set Top to..." and "Set Bottom to..." will set the total Z range relative to the current focused position. The ASI stage focus controller (see below) is a more precise one.

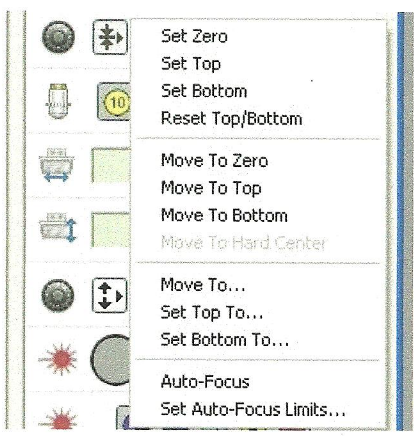


Indicator of objective currently used. The numerical aperture appears when positioning the mouse over each objective icons.



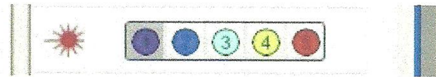
Indicator of the current absolute stage position in X and Y.



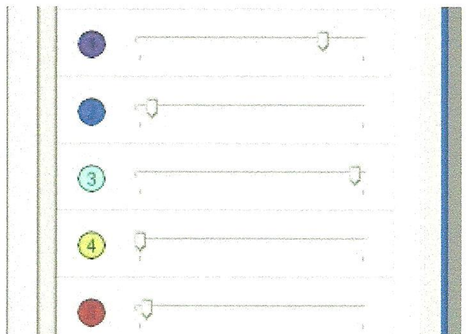


Indicator and control of the ASI high precision focus drive (i.e. the stage is going up and down). "Set Top" and "Set Bottom" will set the absolute Z positions of the Leica focus drive. "Set Top to..." and "Set Bottom to..." will set the total Z range relative to the current focused position.

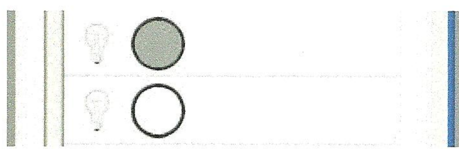
Shutter for the LMM5 confocal lasers.



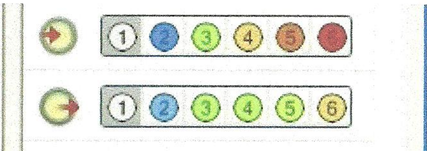
Selection of confocal laser wavelength:
1: 405nm, 50mW (ensure the right dichroic is in place)
2: 491nm, 50mW
3: 440nm, 40mW (ensure the right dichroic is in place)
4: 561nm, 50mW
5: 640nm, 60mW



Control of laser intensity by neutral density filtering. ATTENTION: not necessarily linear (data available on request).



Ludl1 Shutter 2: for the metal-halide fluorescent light source used for wide field fluorescence (e.g. looking at the sample with the eye pieces).
Ludll1 Shutter 3: for the halogen lamp used for bright field, phase contrast, polarization and DIC methods.

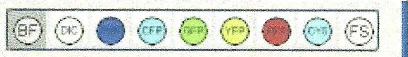


Ludl Filter wheel 1:
1. Empty
2. DAPI 460/50nm (only when the 405/491/561/640 dichroic is installed)
3. FITC 525/50nm
4. Cy3 595/50nm
5. TxRed 620/60nm
6. Cy5 690/50nm

Ludl Filter wheel 2:
1. Empty
2. CFP 470/30nm (only when the 440/491/561/640 dichroic is installed)
3. GFP 515/30nm
4. FITC 525/50nm
5. YFP 540/30nm
6. Cy3 595/50nm

Configuration of light paths

Light paths to be used should be checked at every microscopy session!



The camera is monochrome but a pseudo-color can be assigned to each light path. Right-clicking the light path icon will open an "Edit..." window where a pseudo-color can be chosen.

Once the light paths are configured as required, right-click to overwrite them. Configure one light path per channel.

CONFOCAL LIGHT PATHS

Hardware settings:

-Use the Leica contrast mode "TL/BF".
-The Leica fluorescence turret should go to "empty/laser".
-Choose the proper excitation laser line (e.g. 491nm).
-Set the laser light intensity (e.g. 30%).
-Choose the appropriate emission filter and make sure it is inserted in the light path (see below).
-Set camera exposure (e.g. 300ms).
-Set camera sensitivity, or EM gain (e.g. 100).
-Activate auto-contrast, use 1X1 binning and high-speed read out, deactivate photon-imaging.
-Make sure the LMM5 shutter opens, while the ones for the halogen and metal-halide lamps close.

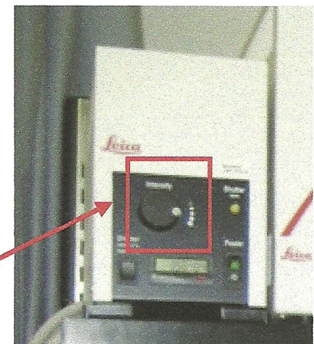
Elements to consider:

-Use the highest intensity of laser that the sample can tolerate without bleaching. Remember the power of each laser is different.
-Use the longest exposure time that is possible. Time-lapse experiments may impose a limit for fast acquisition rates.
-Adjust the camera sensitivity to reach an acceptable ratio of signal to noise. Systematically using the maximum possible gain (250) will put stress on the camera. Set it to reach no more than 50-70% of the maximum grey levels possible (33,000 to 46,000 grey levels). This is rarely achieved in fluorescence imaging, even less in live sample imaging.

TRANSMITTED LIGHT PATHS
Hardware settings:
-Use one of the available transmitted light Leica contrast mode:
Bright field (BF)
Phase contrast (PC)
Polarization (Pol)
Differential interference contrast (DIC)
-The Leica fluorescence turret should go to "empty/laser" (BF and PC), or to "Ana" (Pol and DIC).
-Make sure the emission filter is set to "Empty" (i.e. 100% transmission).
-Set camera sensitivity, or EM gain to 0.
-Set camera exposure (e.g. 50ms).
-Activate auto-contrast, use 1X1 binning and high-speed read out, deactivate photon-imaging.
-Make sure the halogen lamp shutter opens, while the ones for the LMM5 and metal-halide lamp close.

Elements to consider:
-Careful not to saturate the camera (i.e. 65,535 grey levels). If necessary, adjust intensity of halogen lamp from the left side of the microscope stand.

WIDE FIELD FLUORESCENCE LIGHT PATHS
Hardware settings:
-Use the "IL/Fluo" Leica contrast mode.
-Use the Leica fluorescence turret position:
Position 1: DAPI A4 447nm
Position 2: GFP 520nm
Position 3: Cy3 593nm
-Make sure the emission filter is set to "Empty" (i.e. 100% transmission).
-Set the intensity of the metal-halide lamp just enough to see the sample in the eyepieces.



-Set camera exposure (e.g. 300ms).
-Set camera sensitivity, or EM gain (e.g. 100).
Activate auto-contrast, use 1X1 binning and high-speed read out, deactivate photon-imaging.
Make sure the metal-halide lamp shutter opens, while the ones for the LMM5 and the halogen lamp close.

Elements to consider:
-This light source is the most susceptible to cause photo-damage. Use only if it is absolutely necessary. Minimize exposure of samples at all time. Use transmitted light modes to focus on the sample.
-Use the longest exposure time that is possible. Time-lapse experiments may impose a limit for fast acquisition rates.
-Adjust the camera sensitivity to reach an acceptable ratio of signal to noise. Systematically using the maximum possible gain (250) will put stress on the camera. Set it to reach no more than 50-70% of the maximum grey levels possible (33,000 to 46,000 grey levels). This is rarely achieved in fluorescence imaging, even less in live sample imaging.

COMMON FLUOROCHROMES AND FLUOROPHORES

Fluorophore:	Source	Abs (nm)	Ems (nm)
AlexaFluor 405	Kr^{405}, BDL	401	421
AlexaFluor 350	Hg	346	442
EBFP	Kr^{405}	405	447
AMCA	Hg	350	450
DAPI	Hg, Kr^{405}	358	461
Hoechst 33342	Hg, Kr^{405}	350	461
ECFP	Kr^{405}, $HeNe^{458}$, Hg	435	485
Cy2	Hg, Ar^{488}	492	510
MFG	Hg, Ar^{488}	490	516
AlexaFluor 488	Hg, Ar^{488}	495	519
FITC	Hg, Ar^{488}	492	520
EGFP	Hg, Ar^{488}	492	520
Sytox-Green	Hg, Ar^{488}	503	524
EYFP	Hg, Ar^{514}	500	535
Lucifer Yellow	Ar^{458}	458	536
Fluoro-Gold	Kr^{405}, Ar^{488}	380	536
AlexaFluor 430	Kr^{405}	433	541
AlexaFluor 514	Ar^{514}	517	542
AlexaFluor 532	Nd:YAG	532	553
AlexaFluor 555	Hg, $HeNe^{543}$	555	565
Sytox-Orange	Hg, $HeNe^{543}$	543	569
Cy3	Hg, $HeNe^{543}$	550	570
TRITC (Rhodamine)	Hg, $HeNe^{543}$	550	570
AlexaFluor 546	Hg, $HeNe^{543}$	556	573
MTO	Hg, $HeNe^{543}$	554	576
OFP	Hg, $HeNe^{543}$	550	580
RRX (RhodamineRedX)	Hg, $HeNe^{543}$, $ArKr^{568}$	570	590
AlexaFluor 568	Hg, $HeNe^{543}$, $ArKr^{568}$	578	603
mCherry	$HeNe^{543}$	587	610
AlexaFluor 594	$HeNe^{543}$, $HeNe^{594}$	590	617
TexasRed	$HeNe^{543}$, $HeNe^{594}$	596	620
AlexaFluor 610	$HeNe^{594}$	612	628
AlexaFluor 633	$HeNe^{633}$, RDL	632	647
To-Pro 3	$HeNe^{633}$	642	656
MTDR	$HeNe^{633}$	640	662
AlexaFluor 647	$HeNe^{633}$	650	665
Cy5	$HeNe^{633}$	650	670
AlexaFluor 660	Kr^{647}, $HeNe^{633}$	663	690
AlexaFluor 680	Kr^{647}, $HeNe^{633}$	679	702
AlexaFluor 700	FRDL, Xe	702	723
AlexaFluor 750	FRDL, Xe	749	775

AMCA, aminomethylcoumarin acetate
DAPI, 4',6 diamidino-2-phenylindole, dihydrochloride
MFG, Mito fluor green

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