Leica TCS SP5 II Instructions

Leica True Confocal Scanning-Spectral Photometric 5

RCB Imaging Facility
Key number 102 B

Regional Centre for Biotechnology

NCR BIOTECH SCIENCE CLUSTER

DEPARTMENT OF BIOTECHNOLOGY

SOP created on 11 Dec 2012 recent update on 11 Jan 2017

LASER SAFETY & GUIDELINES

Do not defeat interlocks.

Do not open panels.

Do not detach light guides.

Prior doing Confocal Imaging Keep the Temperature of room at 21- 22^0 C.

While during imaging do not disturb both the Microscope & PC Laser table.

Imaging room should be kept dust free.

There should be no vibration or shaker kind of instruments should be running near the Imaging room.

Do not open any external part of the Instrument.

Imaging should be made under Technical Observation only for non certified users.

Make proper Google calendar booking.

Use of cell phone is prohibited inside microscopy room.

Submit duly filled sample submission form prior to experiment.

SWITCH ON PROCEDURE

Only people who have received introduction instrument training and passed certification test can use instrument alone and on holidays are allowed to operate the system! If you need intro please write to **surajtewari@rcb.res.in, sivaram@rcb.res.in, or sjmathew@rcb.res.in**

1. **Check** the microscope if everything looks clean and normal. If not report it in the logbook.

2. **Switch on** all the three green buttons marked as **PC Microscope**, **Scanner Power** and **Laser Power** on the right side under the table are. Switch these on in this order and turn the **Laser Emission key** 90 degrees clockwise.

3. **Switch on** the **fluorescent lamp** (white box on the left side of the microscope desk).

4. **Switch on** the **thermostat** (blue box on the small desk left of the microscope) if you want to do live imaging with temperature control.

1. **Login** to the computer with your account.

2. **Wait** 1 min so the system is fully booted.

3. **Click** on the **LAS AF** software icon on the desktop. Shortly you will see the **LAS** start screen. Check under configuration that **machine** is selected. Press **OK** to start the system.

4. At the end of the starting process it will ask you to initialize the stage. Click **YES**.

5. **Switch on** those lasers that you want to use in the **LAS AF** software under the **Configuration\Laser menu**.

If you switch on the argon laser put it on 11% always. Note that the **405 and argon lasers** need at least 20 minutes warm up time for proper use.

6. The machine is ready to use.

TURN ON HARDWARE

A. Switch on *button pad* – left to right then turn key on.

a). *First* switch on *PC Microscope button*

* You do not have to start the operating system – it starts automatically when you switch on the computer.

b). Log On the Computer Under "USERS"

c). *Second* switch on *Scanner Power button*

   Wait for 2 minutes

d). *Third* switch on *Laser Power button*

*The power supplies and fan of the system have been started.

e). Turn the *Laser Emission key* towards *On-1*

Wait for 2 minutes

START SOFTWARE

*Note :- HARD to SOFT
(Confirm all Hardware's *ON* then go to Software)

Activate the Leica *LAS AF Confocal* software with the icon on the desktop.
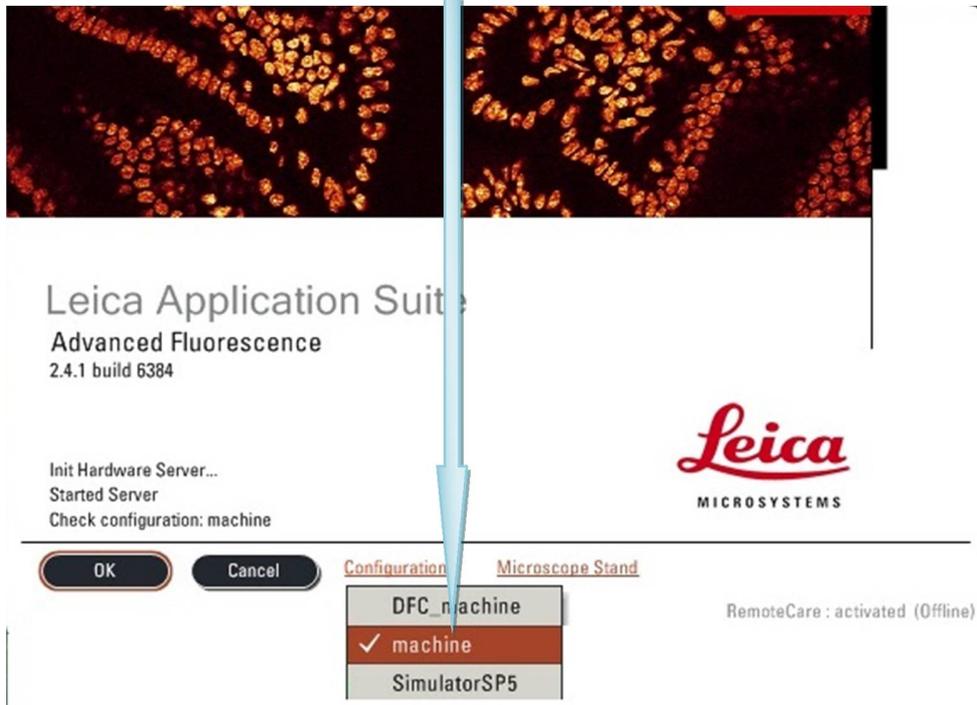

*Before the system start or start of the LAS AF, the stage must be moved downwards, because during initialization, it can come into contact with the objective nosepiece and damage the objectives
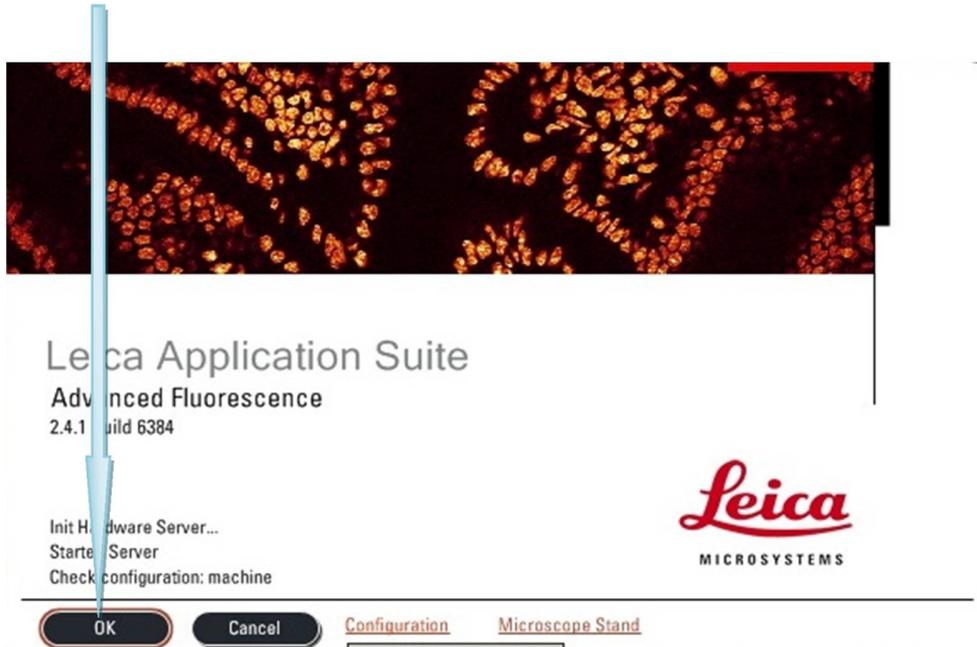
Look for the settings *DMI 6000* in *Microscope Stand*
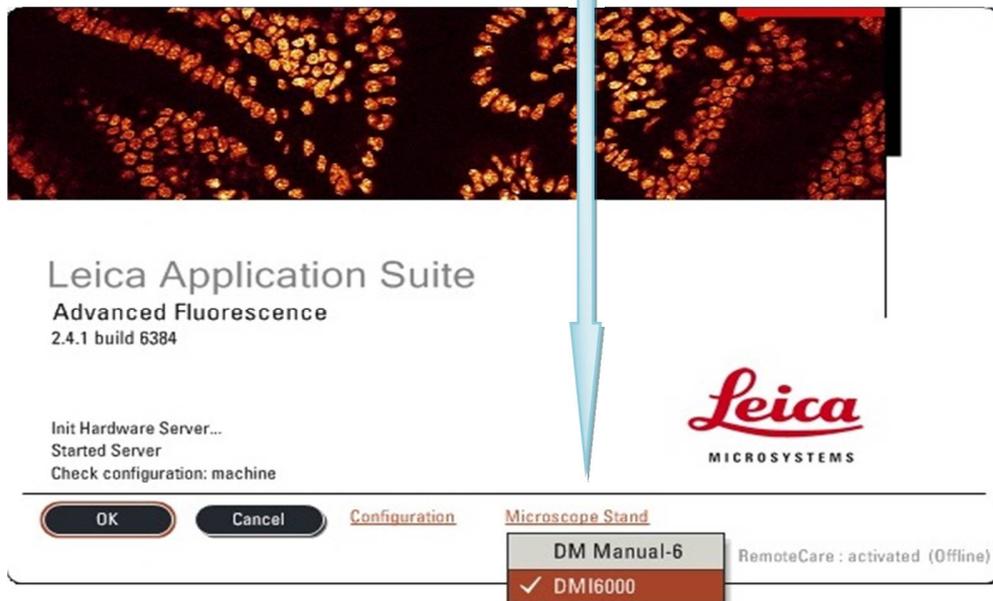
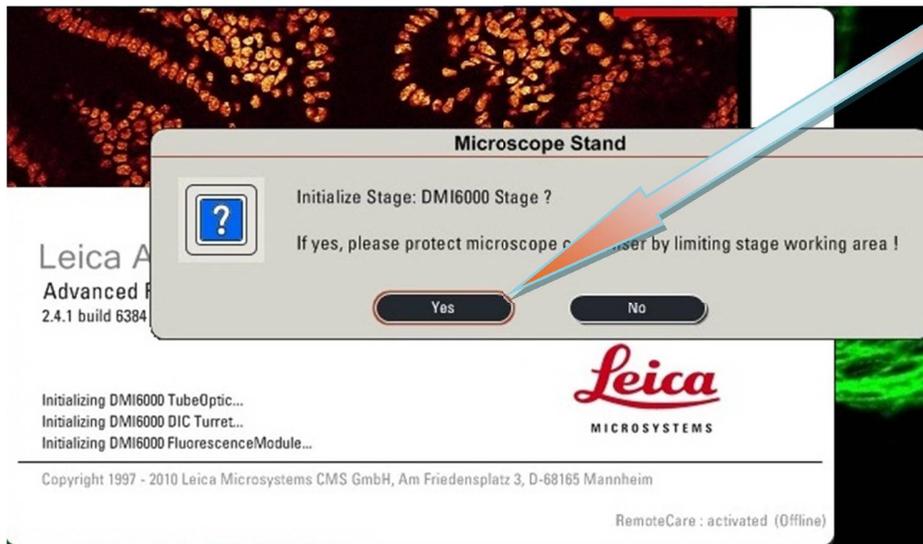Look for the settings *Machine* in *Configuration mode*

Click *OK* in the splash-screen.

After clicking on **OK** button Software will start analyzing the configuration for the Instrument.

Software will ask for Stage Initialization, make sure the stage is moved downward & then press **YES.**


IMPORTANT: **Read below BEFORE proceeding**.
When the **Microscope Stand** windows appears, select **No** unless you plan to use the **Tile** or **Mark and Find** functions for the motorized stage.
NOTE: If you need to initialize the stage, **you must first tilt the transmitted light arm (condenser arm) back from the stage**, because the motorized stage might possibly hit the condenser during initialization. Then click **Yes**.
Once the software is initialized, the screen will pop up & will appear as follows. Where Position 1, Position 3 and Position 5 are PMT and Position 2 and Position 4 as HyD.
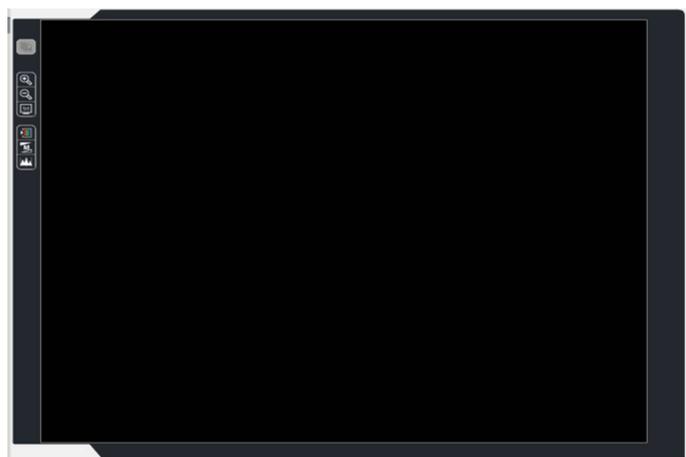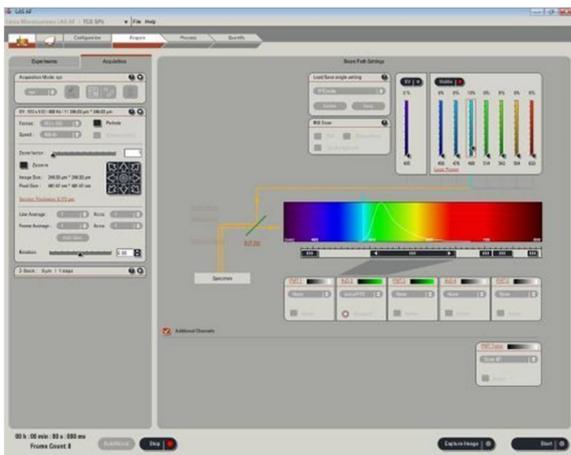
Select the *Configuration* tab and then the *Laser setup* window.
Tick the lasers you require. If you are using   the Argon ion laser, drag the slider to 11 %.
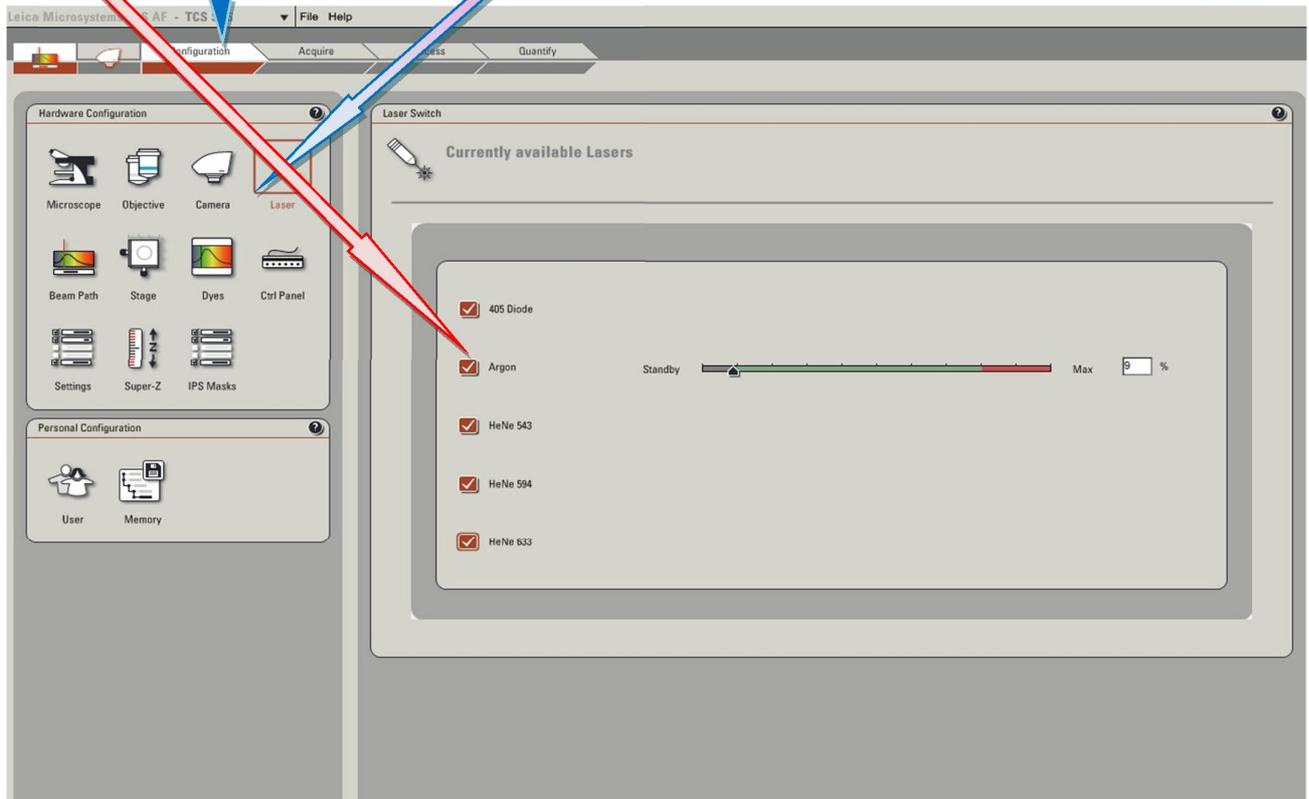
MOUNT SAMPLE

Gently tip the transmitted light arm backwards for easy access to the stage.

Add a small drop of Type F immersion liquid to your sample or the objective and mount your sample. Raise the objective by activating coarse focus on the axis controller unit and focusing up using the microscope's focus wheel until the objective just touches the immersion liquid.

Gently return the transmitted light assembly.
(NOTE: - While moving the light arm do not apply extra pressure or force)

Find sample through eye piece.

Fine

Course

OBJECTIVE

Choose an objective lens from the drop down menu.

|	
10x air 0.40 NA	40x oil 1.3 NA
20x air 0.70 NA	63x oil 1.4 NA
40x air .85 NA	100x oil 1.4 NA

Place the appropriate immersion oil (EXCEPT for the air lenses 10x, 20x and 40x) on the objective lens. Place your sample on the microscope stage. **BE VERY GENTLE**--the stage is very delicate. Position the sample over the lens using the stage controller on the **SmartMove** and focus using the Z axis wheels (either **Fine** or **Course** settings).
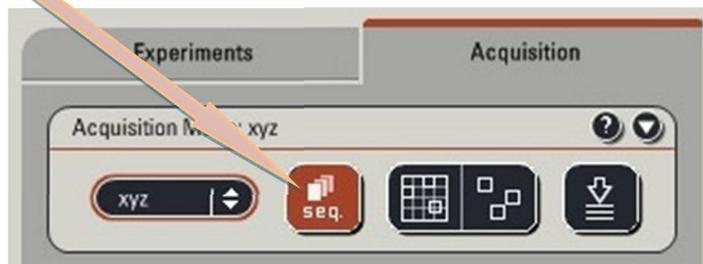
CONFIGURE HARDWARE
A). Dye settings

In the *Acquire panel* turn on sequential acquisition,
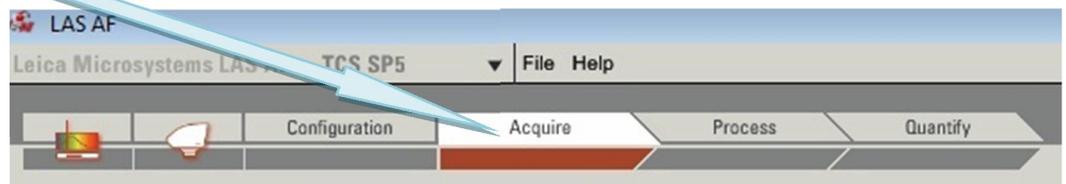
click the *seq.* button on the *Acquisition mode* window. The sequential control panel should now appear at the bottom.
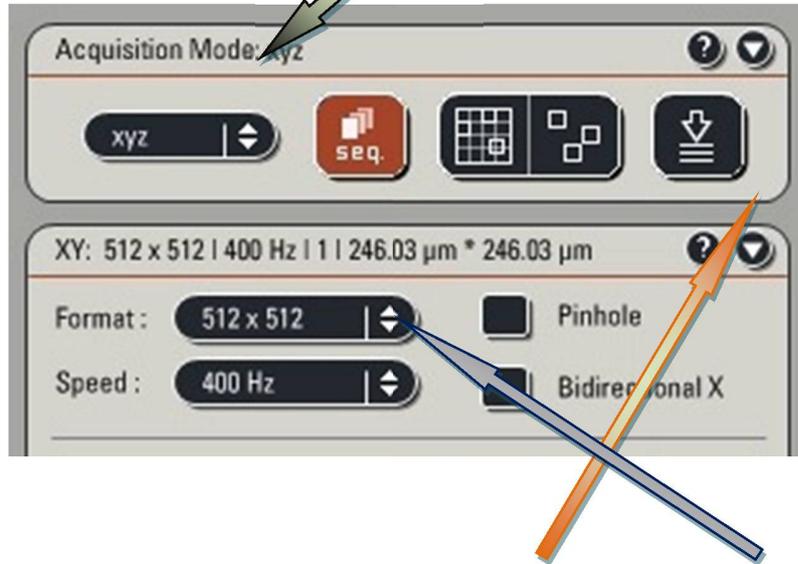

If you already have a sequential acquisition protocol saved you can *load* this now.
If you need to configure a new protocol, add the number of channels you wish to acquire by clicking on the *plus button*.
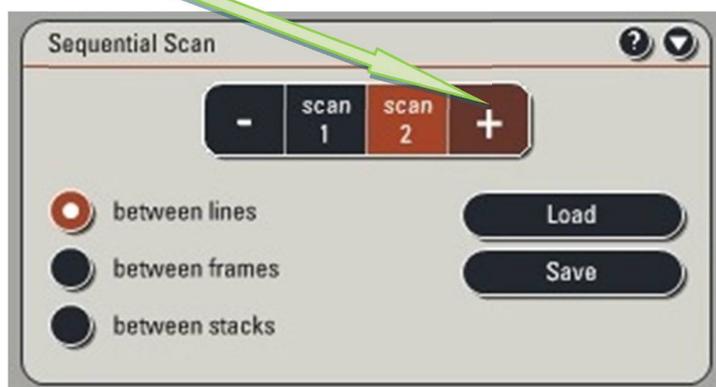
B). Scan speed

While you configure the detectors, set **_Acquisition Mode_** to XYZ.


Set the scan speed to high by activating the **_XY dialog_** and selecting **_400Hz_** from the Speed drop down box.

You can speed scanning further by using the **_Bidirectional X_** scan option although this sometimes results in a de-interlaced image.

This will give you a noisy image but allow faster setting of the detectors with less bleaching of the sample.

C). Pinhole

The pinhole determines the thickness of the "optical section" you acquire. The thinner this is the sharper you image, but the fewer photons in your image (i.e. it will be noisier). The optimal size of the pinhole is determined by the objective's numerical aperture and the wavelength of light you are acquiring.
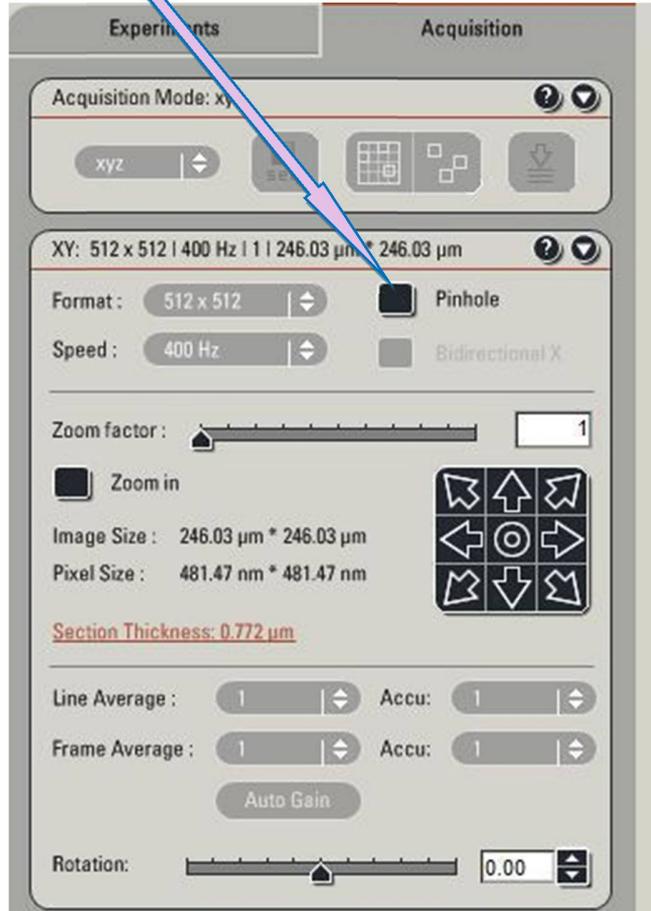
Configuration Window

Visible Lasers:
Argon with multiple laser lines 458, 476, 488, 514 nm
Yellow diode with 546,594 nm
Red diode with 633 nm

UV: 405 diode laser

Beam Path Settings

You can choose a preconfigured setting

Load/Save single setting

FITCwide

Delete   Save

ROI Scan

ROI   Bleach Point

Set Background

UV   Visible

3 %   0% 0% 13% 0% 0% 0% 0%

To open a laser shutter, click on the radio button (red when open)

Laser power is adjusted using the

405   458 476 488 514 543 594 633

Laser Power

Control Panel

Spectrometer

Objective: HC PL

RSP 500

[nm]   400   500   600   700   800

Choose an objective

Specimen

PMT 1   HyD 2   PMT 3   HyD 4   PMT 5

None   Leica/FITC   None   None   None

Active   Standard   Active   Active   Active

Additional Channels

Spectral detection (emission) range is adjusted here

There are 2 HyD detectors and 3 photomultiplier tube (PMT). Check the box to activate the laser

PMT Trans

Scan-BF

Active

Transmitted light detector
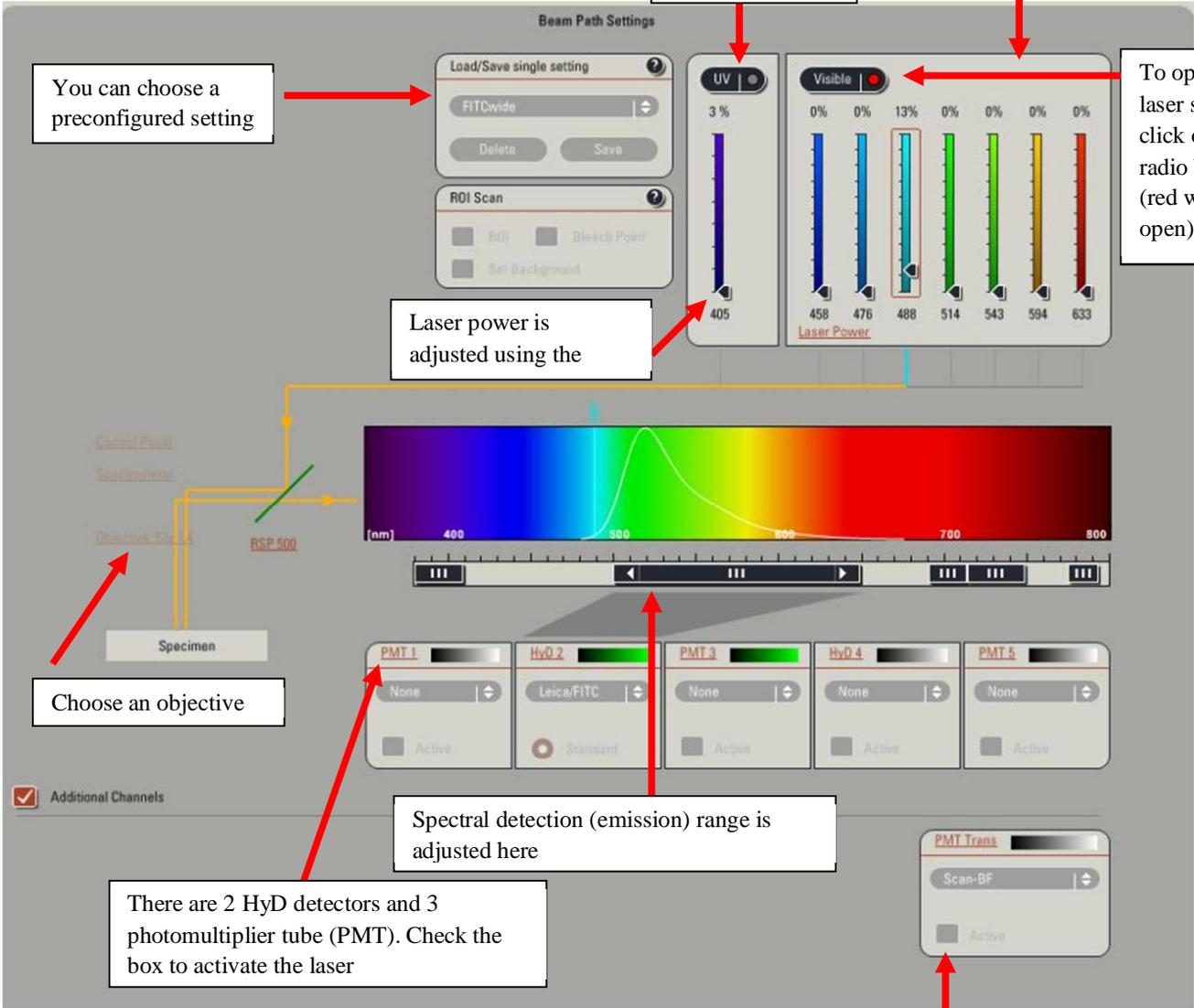
ADJUST DETECTOR SETTINGS

A). Prepare display

To aid adjustment of the detectors, the display should be set to show the *Merged image* and to use the "*Quick LUT*" look-up table.

Click the Quick LUT button until it cycles to the range set LUT.
This LUT will display bright-green when the grey level equals zero and bright blue when it saturates. Where blue means over-saturated area immediately reduce AOTF range. Green means undersaturated area with HyD there will be great signal to noise ratio so green range will not be a problem.

B). Adjust Offset

When there is need to change the offset or gain, always click Live and after adjustment click Stop. The adjustment should make in such way so that they should not make the area under observation go Bleach out. When not in observation go Stop.

NOTE: The spectral range should always be at least 10 nm greater than the excitation laser line.
Example: If using the 405 nm laser, then the minimum in the spectral range should be at least 415 nm.

Adjust the focus until you have the brightest focal plane of interest.
Click on the image pane of *channel 1* to highlight it.
While scanning, adjust the
*Offset dial (Only For PMT)*so that there
  Green pixels in the background of
  channel 1. Click on *channel 2*
  and repeat this. With HyD there
  will be no offset change available.
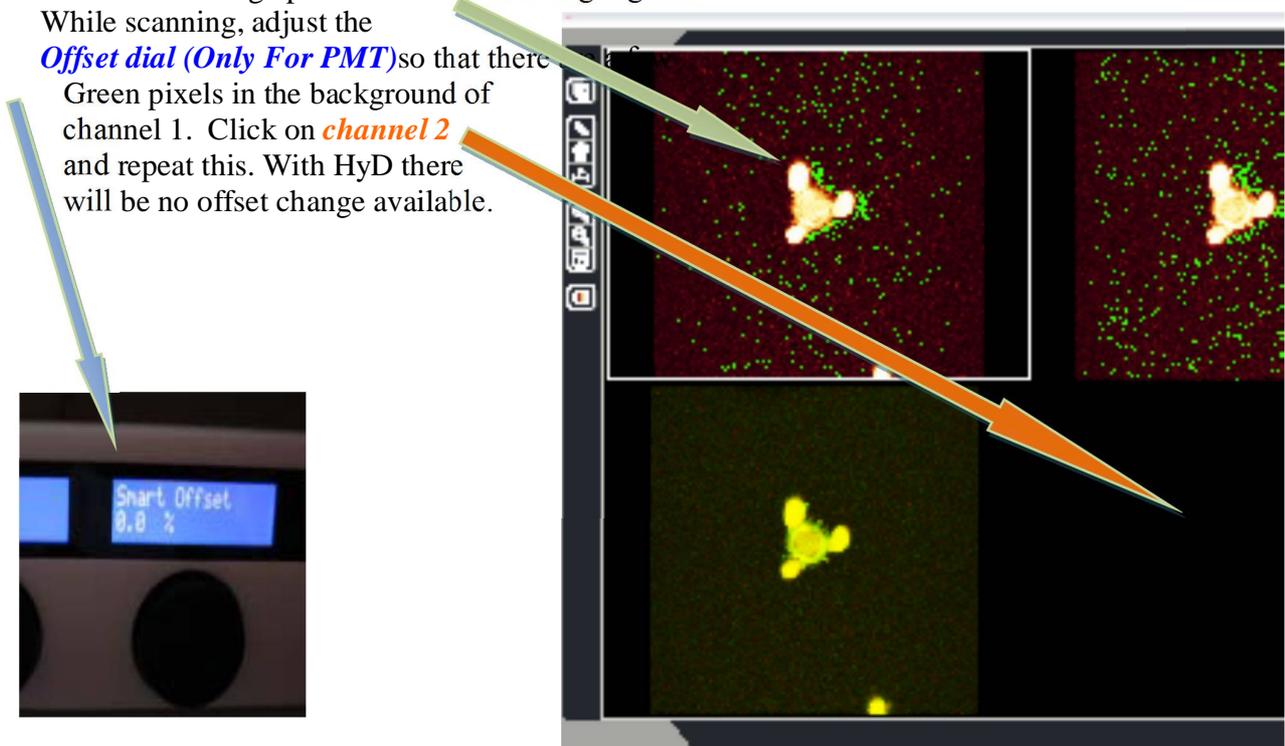
Click the *Live button* to scan. This will now change to the *Stop button* while live.

C). Adjust Intensity

The intensity of the image is dependent on two things at this point: the number of photons generated (*laser power*) and the amount they are amplified by the detector (*detector gain*). The optimal settings need to be determined empirically. Adjust the intensity with these two parameters until there are a few speckles of bright blue pixels indicating that you are using the full range of the detector.
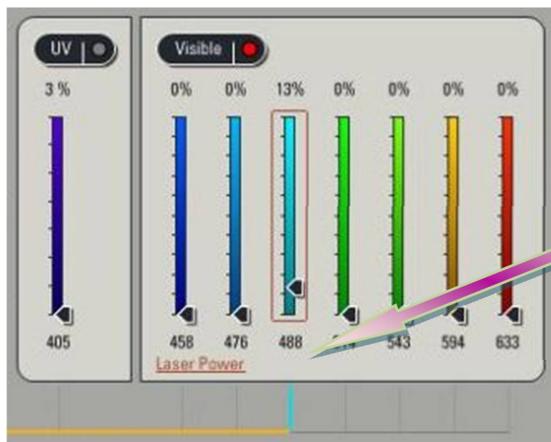
D). Detector Gain

You can increase the signal amplification using the *Gain dial* but this will increase noise. The gain *dial* will max out at 1000 V but extra gain up to 1250 V can be achieved using the software control. If you increase gain a long way, you may need to reset the offset. Try not more than 700 V.
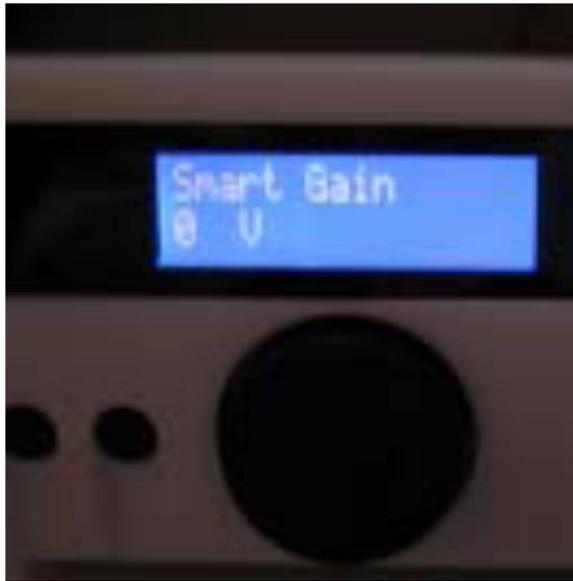
E). Laser Power

Visible continuous wave (CW) lasers. You can increase AOTF values in %
the number of photons generated by adjusting the *laser powers* but this will increase bleaching
and will cause overloading in detector. Try keeping lower values to begin with like 0-5%.

Ultra Violet (UV) lasers . You can increase the UV laser as per the requirement.

Set the **laser power** with the slider. Start with 3%.
REMEMBER: The HyD detectors are very sensitive.
Make sure all the other lasers are at 0% power.

For imaging use PMT first. If image comes with PMT do not try to go for HyD.
If HyD is needed in cases for imaging then lower down AOTF values to 5% and the open LUT to see saturation level. If oversaturation appears reduce AOTF
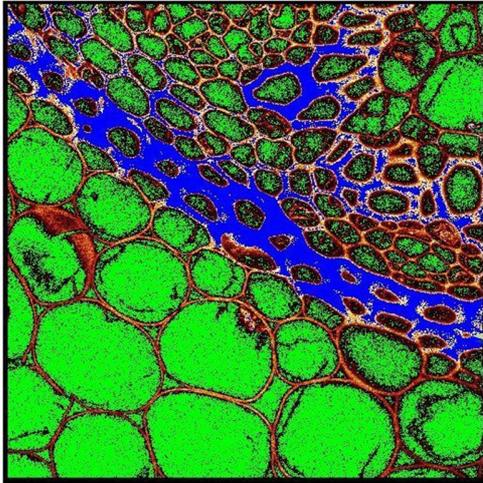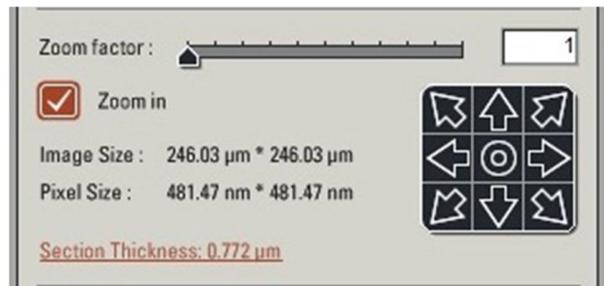
saturated

SET FINAL SCAN PARAMETERS

A). Frame size

Before setting the frame size, crop the image to include only the field of view of interest. Select *Zoom in* from the XY panel, then select the rectangular *region of interest (ROI) tool* and drag this around the area you want to acquire.

You can resize and reposition this ROI.

Zoom factor :   1

☑ Zoom in

Image Size :   246.03 μm * 246.03 μm

Pixel Size :   481.47 nm * 481.47 nm

Section Thickness: 0.772 μm

Activate Live scan to ensure you have the correct field of view selected, then stop acquisition. The frame size should be set to ensure optimal *pixel size*.

Pixels smaller than the optimal will result in over sampling, increased bleaching, increased noise and no extra spatial information.
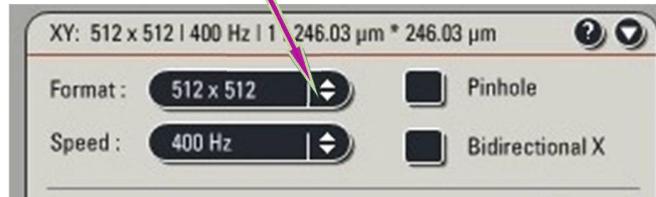Pixels larger than the optimal will result in lower spatial resolution. This may or may not be important to you.

Objective Optimal pixel size (nm) with 488nm laser
63× 1.3NA   100
20× 0.7 NA   200

Adjust the frame size by clicking the *Format* drop down box and selecting *More*.

SCAN SPEED

The noise in a confocal image is primarily intrinsic photon shot noise. The Signal to noise ratio is proportional to the number of photons sampled. So you get less noise if you sample more photons. Reducing the speed of the scan effectively increases the photon sample time per pixel – the "pixel dwell time". It does reduce the frame rate so a slow scan speed is not suitable for adjusting the detector settings or high speed biological events.

For fixed samples where maximum resolution is required, set the scan speed to *100Hz.*

AVERAGING

Another way to improve the signal to noise is to sample and average each pixel a number of times. The microscope can be configured to average a number of frames and/or lines. Or for weak signals, these values can be added to 'accumulate' the signal.
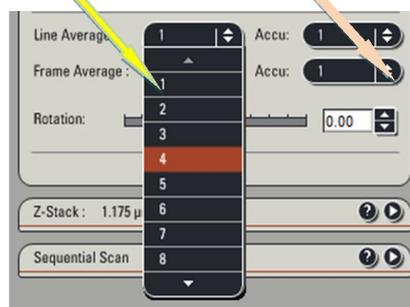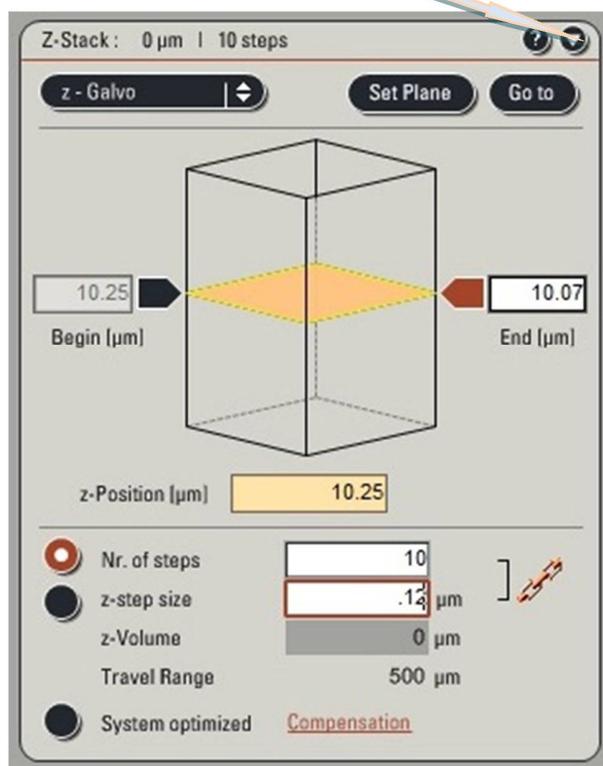
NOTE: Averaging removes "noise" detected by the PMT. Generally, less averaging is required when the Gain is set low.

ACQUIRING A Z-SERIES
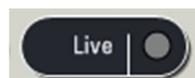
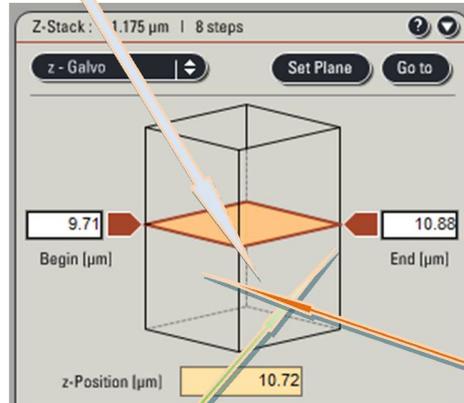Set Z-range First, change the Speed to *400Hz* to facilitate setting the z-range.

Activate the *Z-stack* drop down window

Activate *Live* acquisition

.

Click the beige *plane indicator* to select it. It will get an orange border one it is highlighted

Use the mouse wheel to scroll to the top of the sample. Then click the ***Begin*** block-arrow which will then enter the z-position.
Select the *plane-indicator* again and scroll down until the bottom of your sample appears in the live image window. Click the ***End*** block-arrow to set the end of the z-range.

Set Z-step

By default, the software calculates the optimal z-step and the number of steps required for the range you have set.
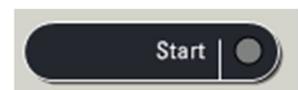However, often, we do not need to sample with such high frequency.
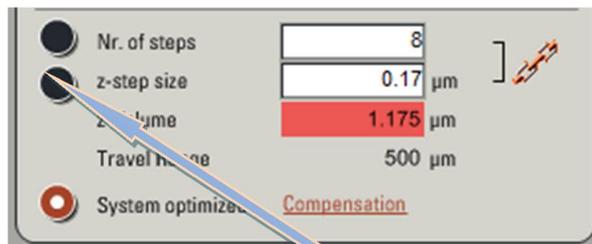
To override the default settings, select the *top radio-button* and enter the step size, or number of slices you wish to acquire.

Acquire Z-series
Change the scan speed back to your original slow speed then select ***Start***.

Your z-series will then be sent to your experiment and need to be saved.

SEQUENTIAL SCANNING

To minimize the potential for cross-talk between fluorophores, each channel can be acquired in sequence. This means that when you are collecting the red emission, the green dye is not being excited so does not appear in the red-channel.
This is of critical importance for colocalisation studies but does cause problems with live cells as the sample may move between channel acquisitions. It is best to acquire channels in the order 'longest-wavelength-first", i.e. red, then green, then blue. Channels can be obtained sequentially in a number of ways.

Between lines is quickest and allows for fewer issues with movement artifacts, but does not allow radical changing of the filter settings.

Between Frames allows filter sets to be completely reshuffled between channels for maximum cross-talk prevention, but has a significant time delay between channels and prone to movement artifacts.

Between stacks. When acquiring a sequential channel z-series, the delay between frames and duration of acquisition can be very long if you change settings between frames (i.e. each z-slice).
This option speeds acquisition of z-stacks but can result in movement artifacts and z-registration issues. These registration issues can arise if the z-focus does not return to exactly the same position for slice 1 of channel 2. This can cause errors, especially in colocalisation or ratio-metric analysis.

Set channel settings

Select the first channel and configure as you would a simultaneous acquisition (Section 5).
Click Scan 2 button and repeat for your shorter wavelength dye. Repeat for all channels.
Adjust the final scan parameters.
Save these settings to reuse them next time using the *Load* button.

REMEMBER: The minimum in the spectral range should be 10 nm greater than the 405 nm laser line.
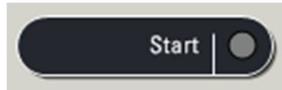
FOR HyD DETECTORS, THE SMART OFFSET IS ALWAYS 0.0%, so you can only adjust the gain.
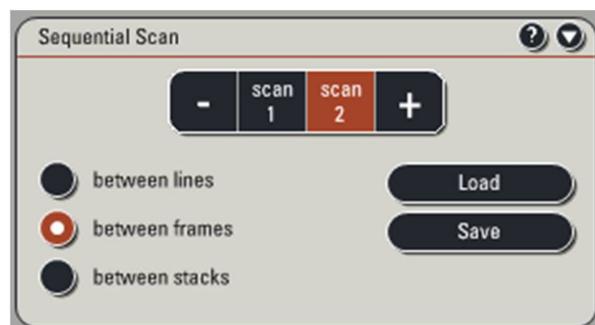
EXECUTE EXPERIMENT

A). Acquire

To acquire a single XY image (actually XY as each pixel has a finite z-depth) click the *Start* button.


This will put the XY image in the *Experiments tab*.

B). Save

Your data is saved in a single experiment file (*.LIF). This file contains the multiple image series (xy, xyz, xyt etc.) that you acquire whenever you hit the *Start* button.
Save your experiment by right clicking it and selecting *Save Experiment As* from the menu.

Rename the current image (series) by right clicking it and selecting rename.
Subsequent acquisitions will be added to this Experiment, but not automatically saved.
You need to right click the series rename to rename it something more memorable than "Series 2".
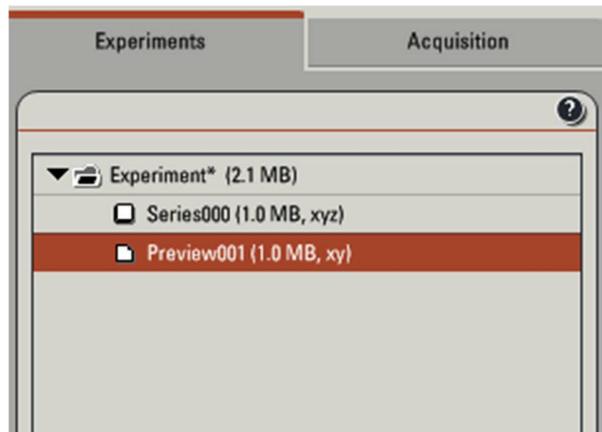
Then right click select *Save Experiment…* to save the data.

COPYING DATA

The saved data then can be copied into cd/dvd's. Data should be properly labeled and stored with proper entries.
Avoid use of USB in all condition (*This probably can cause serious damage to the installed software for imaging*)**.**

SWITCH OFF PROCEDURE

When you are finished with your study do the following:

1. **Save** your **files** to your folder. It is recommended to save the data file (.lif) which contains all the settings of your experiment. You can reuse these settings later when you do imaging with similar samples.

2. **Clean** all the **objectives**, the **condenser** and the **stage.**

IF YOU DO NOT CLEAN THE OBJECTIVES PROPERLY, YOUR MICROSCOPE PRIVILEGES WILL BE TAKEN AWAY.

3. **Swing in** the **lowest magnification objective.**

4. **Close** **LAS AF** software and log-out from your windows account. If you are not the last one of the day (check in the booking calendar!), leave all the lasers ON.

If you are the last user of the day (check it in the booking calendar), follow with these steps to shut down the microscope:

5. **Switch off** **all lasers** in the **LAS AF** software (Configuration\Laser, uncheck the boxes) before close the software.

6. **Switch off** the **thermostat** (blue box on the small desk left of the microscope).

7. **Switch off** the **fluorescent lamp** (white box on the left side of the microscope desk).

8. **Turn the** **Laser Emission key** anti clockwise to off. Wait 5 min while the lasers cool down. You can hear when the ventilation switches off automatically.

9. **Shut down** **Laser Power, Scanner Power** and **PC Microscope** buttons in this order. Do not switch off the **Laser Power** button until you can't hear that the laser ventilation turned off (or if you waited 5 minutes)!

10. *Use the **logbook** and write in any problems and comments occurred during your microscope session! If you signed the logbook and did not report any problem, it means you left the machine in perfect condition.*

11. **Switch off** all the lamps in the room and close the door submit the keys back.