

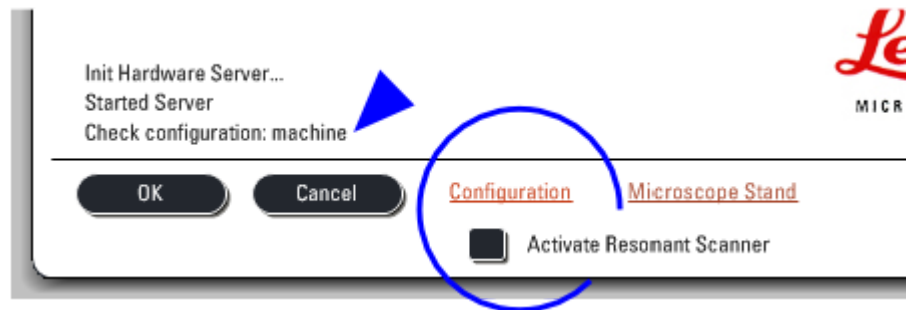
# Instructions for the Leica SP5 II laser scanning confocal microscope

Content: [Check-in and Start up](#) • [Set up acquisition parameters](#) • [Optimize acquisition parameters](#) • [Acquire a z-stack](#) • [Sequential scan](#) • [Check out](#) • [Appendix](#)

## Check-in and Start up

1. Main Switch Board: switch on PC Microscope, log on and wait until Windows XP is fully loaded
2. Main Switch Board: switch on Scanner Power, wait 5 S
3. launch LAS AF (desktop shortcut), select the desirable Configuration and scanner mode, then click ok

*Note:* Usually, Configuration is Machine and Activate Resonant Scanner is unchecked. However, if the previous user has selected simulated SP5 or used resonant scanner, these will become the default when start up, therefore please check carefully before clicking OK.

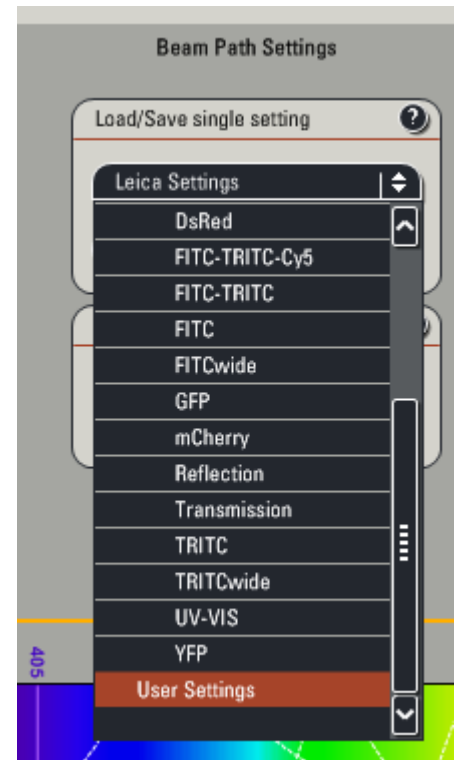


4. make sure the condenser is in the "up" position, click Yes to initialize the stage when prompted
  - this enables Mark and Find, and Tile scan
  - stage is configured to return to the position before initialization
5. Main Switch Board: when the software finish loading, switch on Laser Power and turn the key clockwise to On-1 to enable Laser Emission
6. in LAS AF, click on the Configuration Operating step on top left to bring up Hardware Configuration
  - Laser: check to activate lasers that will be used for the session
 

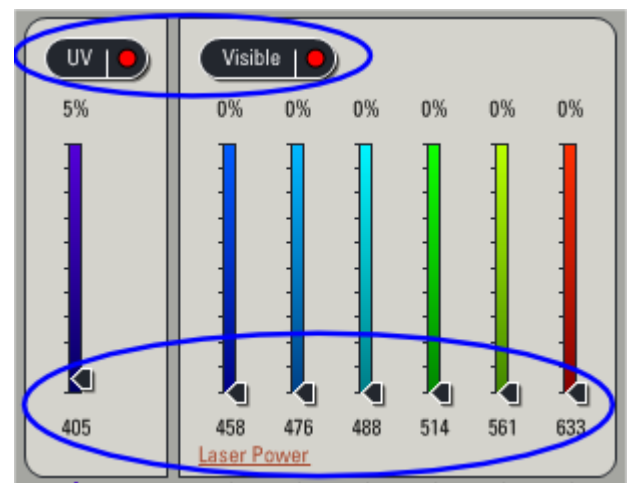
*Note:* If you need the Ar laser, set the power to 20 %. You can use higher power which will shorten the life span of the laser; but one should never go above 80 % into the red region.
  - (optional) Settings > Resolution > Bit Depth: select 12 Bit for 4096 grey levels, the maximum this system can offer

# Set up acquisition parameters and acquire an image

1. Beam Path Settings in the central Work area: there are 3 ways to configure excitation and emission parameters
  - A. use a preset
    - i. click on Load/Save single setting
    - ii. select a Leica or User Settings (for multichannels, the default is simultaneous scan, see [below for sequential scan](#))



- B. reuse a setting from a previous scan
  - i. open a previous experiment
  - ii. in the Experiments tab, click to select the desirable scan
  - iii. right-click to choose Apply Setting or hit Apply button at the bottom
- C. assemble from scratch
  - i. set laser power
    - activate the shutter
    - set power level of the desirable laser line, 5 % is a good starting point

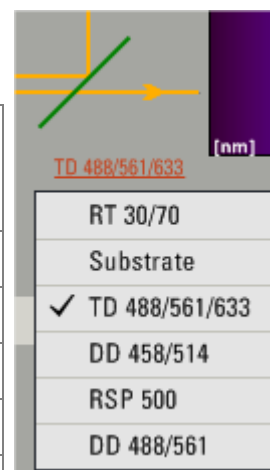


## ii. select the beam splitter (dichroic)

## selections for different laser lines

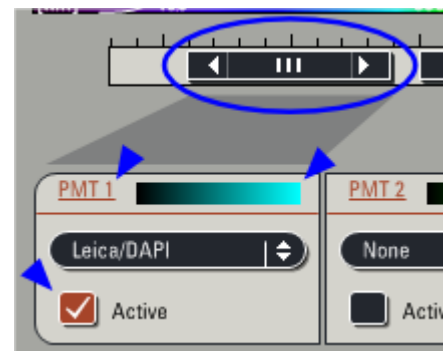
| beam splitter<br>\<br>laser line (nm) | RT 30/70 for<br>backscatter<br>or reflection | Substrate | TD<br>488/561/633 | DD<br>458/514 | RSP<br>500 | DD<br>488/561 |
|---------------------------------------|--|-----------|-------------------|---------------|------------|---------------|
| 405                                   | ✓  | ✓         |                   |               |            |               |
| 458                                   | ✓  |           |                   | ✓             | ✓          |               |
| 476                                   | ✓  |           | ✓                 |               | ✓          | ✓             |
| 488                                   | ✓  |           | ✓                 |               | ✓          | ✓             |
| 514                                   | ✓  |           |                   | ✓             |            |               |
| 561                                   | ✓  |           | ✓                 |               |            | ✓             |
| 633                                   | ✓  |           | ✓                 |               |            |               |

spectra or details for the beam splitters are in the [Appendix](#)



## iii. setup the detector

- select the emission band: drag the lower and upper bounds or double click the slider to enter the begin and end wavelengths  
*Caution:* Drag the sliders slowly or else they will jam!
- click on PMT n to adjust PMT Gain\* and Offset\*; good starting values are 800 and 0, respectively
- select a LUT as default
- activate the PMT



2. select the Acquire Operating step on top left, click the Acquisition tab to set up the data format

A. Acquisition mode panel

- defaults to xyz simultaneous scan
- seq. button enables **sequential scan**
- Tile scan, and Mark and Find
- Best Focus

B. XY panel (click ► on upper right corner to expand)

- Format: frame resolution defaults to 512x512 pixels, max 8192x8192 pixels
- Speed: line frequency defaults to 400 Hz, scan area will be reduced above 700 Hz, max 2800 Hz
- Zoom\* and panning
- Average and Accumulate
- Scan Field Rotation\*
- Pinhole\*: default is 1 AU and displays in  $\mu\text{m}$

3. acquire an image (buttons in the lower left pane of LAS AF)

- Live: scan, apply accumulate if set
- Capture Image: acquire an image at the current z position, apply both average and accumulate if set
- Start: acquire all configured scans, e.g., z-stack, stage positions, tile scan, time points, spectral series etc.

\*these parameters are also adjustable via the Smart Panel with the default configuration

## Optimize acquisition parameters

1. center your prep in the field of view with either BF or FLUO (see Modes of operation in the [Appendix](#))




2. to toggle on scanning, click Live or use the button on the left side of Smart Panel

*Caution:* if there is no signal in the viewer display Window on the right, DO NOT move the stage controls (XYZ) or increase the laser power; try the following

- i. with BF or FLUO, make sure object of interest is still in focus
- ii. open up pinhole to increase the slice thickness
- iii. raise PMT gain
- iv. lastly, increase laser power

3. bring the object of interest into focus, adjust PMT gain, offset, and laser power to obtain an image with good dynamic range

*Note:* the "glow-over, glow-under", Glow (OU) LUT which displays 0 intensity in green and saturation in blue (255 for 8-bit or 4095 for 12-bit), facilitates this setup

- i. top left side of the viewer display Window: activate the Glow (OU) LUT via the Quick LUT button which cycles through the default , Glow (OU) , and grey  LUTs
- ii. adjust offset so the pixels in the area you think should be darkest just turn green, usually around -0.2 %; increase the sensitivity of the control panel offset knob may help
- iii. vary the PMT gain or laser power so a few pixels in the brightest object of interest just turn blue



4. with multichannel simultaneous scan, always check for cross-talk between channels by dropping the laser power of the suspected channel to zero; if cross-talk does present, use **sequential scanning**

# Z-stack

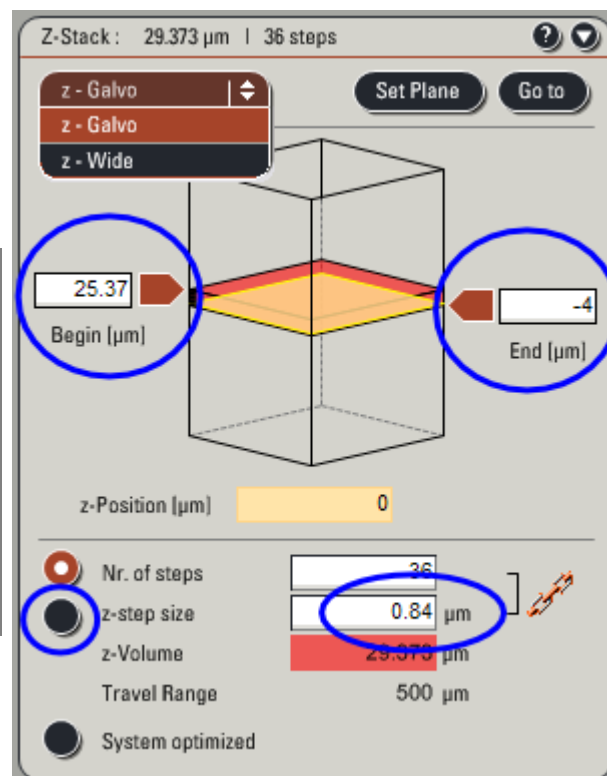
- Z-Stack panel
  - focus control is selectable: z-Galvo (default) or z-Wide
  - focus (z-Position) can be adjusted with the microscope controls, Smart Panel and also via software (see z-drives in the [Appendix](#))

z-drive controls

| controls \ z-drives | microscope focus knob* | Smart Move* | Smart Panel | range                 | min. step size |
|---------------------|------------------------|-------------|-------------|-----------------------|----------------|
| z-Galvo             |                        |             | ✓           | $\pm 250 \mu\text{m}$ | 42 nm          |
| z-Wide              | ✓                      | ✓           | ✓           | $> 4 \text{ mm}$      | 49 nm          |

\*the objective turret i.e., focus is always adjustable with the microscope focus knob and Smart Move, regardless of the z-drive selection in the software


- Acquire a z-stack
  1. in Live mode, move deeper into the prep (z is more positive) to where the stack should start, click Begin to register this z-Position (black: unset, red: set)
  2. move towards the outside of the prep to where the stack should stop, click End to register this z-Position
  3. stop scanning, click the z-step size button and then enter the appropriate slice thickness (check setting slice thickness in [Appendix](#) for recommendations)
  4. select the number of passes to average in the XY panel
  5. click Start (lower middle) to collect the stack



# Sequential scanning

1. set up a simultaneous scan for the fluorophores needed with regard to laser power, beam splitter, emission band, and PMT settings; activate one laser line and detector combination at a time to find out the optimal acquisition parameters



2. click the  button on Acquisition Mode on the upper left to open the Sequential Scan panel
3. select between lines, -frames, or -stacks (see *useful tips* below)
4. click the + button to add as many scans as needed
5. for each scan
  - drop the unwanted laser power to 0 %
  - select the appropriate beam splitter
  - deactivate the unwanted PMTs
6. (optional) save the setting

## *useful tips:*

- if you use different beam splitters, have overlapping emission bands, apply different number of passes in average or accumulate, or need different size pinhole for different scans, you will need to use the slower "between frames" or "between stacks"
- with "between frames" or "between stacks", keep hardware changes to a minimum between scans will ensure fast and smooth operation e.g., use the same emission band for the same PMT in each of the scans
- each scan can have multiple simultaneous channels e.g., adding the transmission detector, or have 2 simultaneous channels that do not show any cross-talk
- the function buttons act differently in different sequential scan mode

## display behavior of the function buttons in sequential scan mode

| buttons<br>\<br>scan mode  | Live                    | Capture Image            | Start             |
|--|-------------------------|--------------------------|-------------------|
| between lines  | all scans               | all scans                | acquire all scans |
| between frames   | currently selected scan | currently selected scan* | acquire all scans |
| between stacks   | currently selected scan | currently selected scan* | acquire all scans |
| *to obtain all scans at the current focus (z-position), switch to xyt mode, set frame to 1, and then click Start |                         |                          |                   |



# Stage automation

*Caution:* whenever the Tile Scan or Mark and Find window is launched

- i. any tiles or points marked will be processed when you click Start
- ii. the grey area represents the stage, a left mouse click will cause the stage to move to that specific location and can be dangerous for any objective lenses except the 10×

- Tile Scan





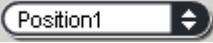
1. click  to toggle displaying the Tile Scan window
2. check that Scan Field Rotation is set correctly (see [relevant details](#) below)
3. go Live
4. move the prep/stage to the left boundary of the region of interest, focus
5. click  to mark the current position
6. repeat the last 2 steps for the top, right, and bottom boundaries, the software will calculate the matrix needed

*Note:* Marking any points outside the established coverage area will expand the matrix but marking any points inside will not change anything. Unfortunately, there is no provision to save the points here or apply any points already saved from Mark and Find.

7. move about to make sure the coverage is sufficient, especially with multichannel acquisition
8. setup Z-stack, configure Average etc., as needed
9. click Start to acquire data

- Mark and Find

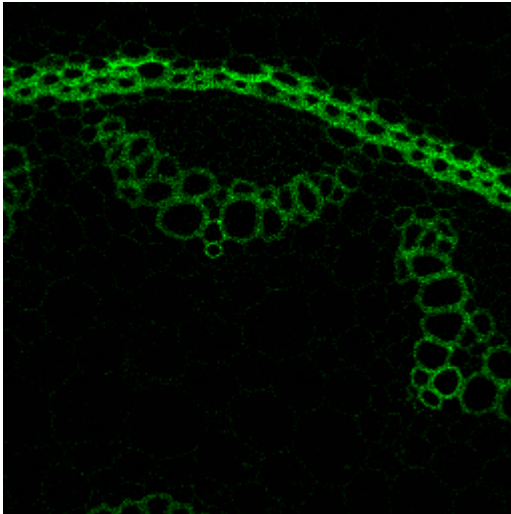


1. click  to toggle displaying the Mark and Find window
2. if there are existing coordinates, you can save or clear them first
3. go Live
4. move the prep/stage to the desirable area and focus on the object of interest
5. click  to mark the current position, which will be listed as positionN
6. repeat the above 2 steps to mark more positions as needed, i.e., positionN+1, N+2... etc.
7. you can go back to any position by selecting it from the drop down list 

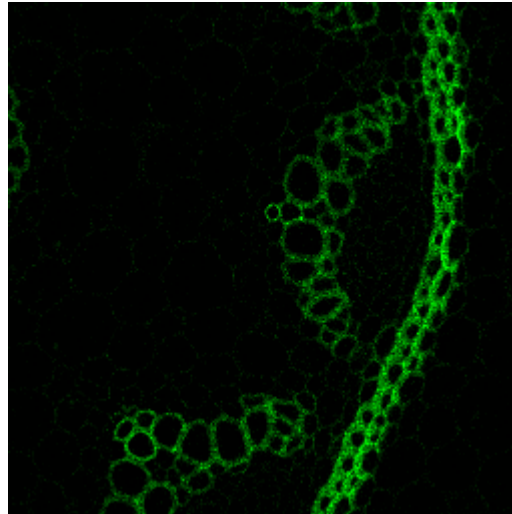
- *relevant details*

- for tile scan, current rotation calibration for stitching is  $-0.19^\circ$  ( $-0.18^\circ$  is also OK) with a 10% overlap
- tile scan order is row-wise in the viewer display window, starting from the lower left, which corresponds to column-wise starting from the lower right as displayed in the Tile Scan window

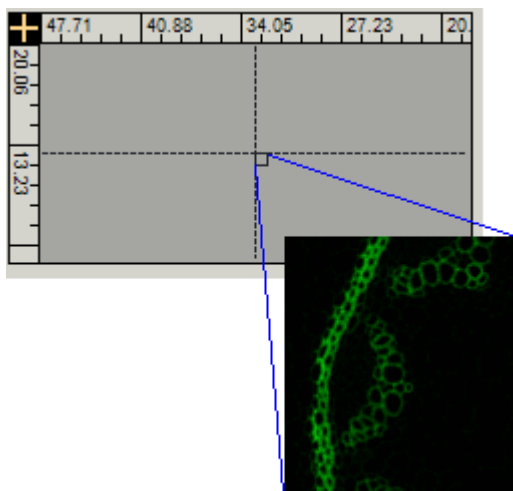
*Note:* The orientation of the specimen as shown in the viewer display window (1) is rotated when compared to that as observed through the ocular of the microscope (2) or represented in the Tile Scan or Mark and Find window (3). Confusing? Indeed!



1. viewer display window



2. at the microscope  
viewer display windows rotated  $90^\circ$  right



3. Tile Scan or Mark and Find window  
viewer display windows rotated  $90^\circ$  left

- coordinate system
  - values (mm) displayed in the window denotes the upper right corner of the image in the viewer display window
  - upper left of the stage is 0, 0
  - lower right of the stage is 126, 82



# Check out

1. lower the turret, remove your prep; if applicable, remove oil/immersion fluid on immersion objectives
2. switch to the 10× objective
3. if there are users signed up within the next 6 hours do the following, otherwise go to the next step
  - i. save your experiments and quit LAS AF
  - ii. copy your data to external storage media or network file servers, as needed
  - iii. submit your usage using the google form and log out of Windows
4. if no one signed up within the next 6 hours, please proceed to shutdown everything
  - i. save your experiments and quit LAS AF
  - ii. Main Switch Board: turn the key counterclockwise to Off-0 to disable Laser Emissions
  - iii. Main Switch Board: wait 10 s, switch off Scanner Power
  - iv. copy your data to external storage media or network file servers, as needed
  - v. submit your usage using the google form
  - vi. shutdown Windows XP
  - vii. Main Switch Board: after computer has shutdown, switch off PC Microscope
  - viii. Main Switch Board: when the Argon laser cooling fan stops, switch off Laser Power
  - ix. turn off FLUO, if applicable
  - x. put the cover back on the microscope
5. clean up the work area

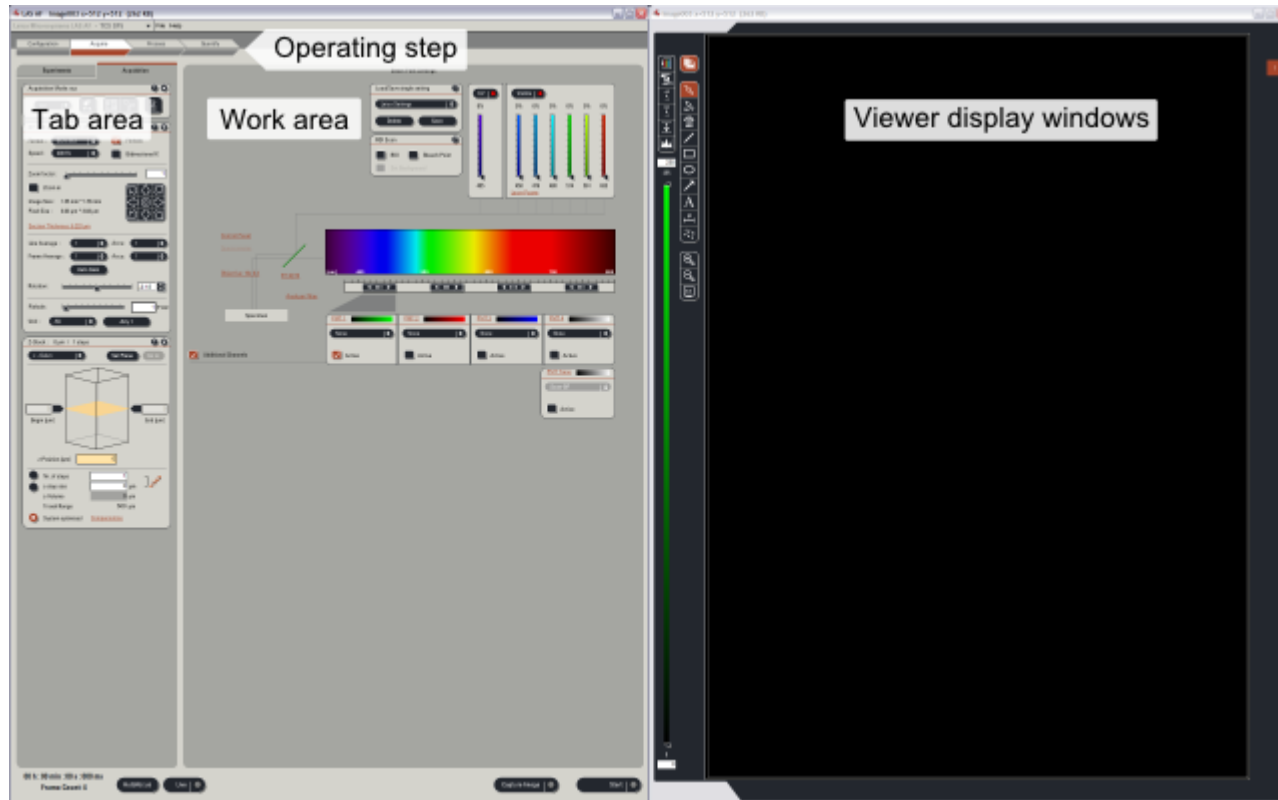
# Appendix

## Content

- DMI 6000 inverted microscope: **basic controls** • **modes of operation** • **using immersion objectives**
- **beam splitter spectra** • **working with lif files** • **scanner**

## software overview and layout

### LAS AF user interface



## DMI 6000 inverted microscope

### Controls

- focus knob on either side of microscope (z-Wide)
- commonly used functions are controlled by 5 sets of buttons
  1. left rear variable function buttons
    - CHG TL : switch to transmitted light, TL and rotate through different modes TL\_BF (bright field), \_DIC, \_POL (polarization)
    - CHANGE CS: toggle between confocal SCAN and the last selected TL mode
    - COMBI : epifluorescent + DIC
    - Z COARSE Z FINE: toggle z control
  2. left front: Illumination Manager
    - TL/IL: toggle between TL and IL (incident-light i.e., epifluorescence)
    - AP: aperture diaphragm for TL



- INT : light intensity, press both together to toggle between Coarse and Fine mode
- FD: field diaphragm for IL

## 3. front

- top row switches should not be activated, or else it may block viewing through the binocular, to rectify
  - press the leftmost button to select binocular
  - press the rightmost button to deselect the supplemental magnification changer
- SHUTTER: for epifluorescent illumination
- bottom 6 buttons: for selecting specific cube (DAPI, GFP, DsRed) for epifluorescence; press the middle 2 buttons together to display the programs assigned to the variable function buttons



## 4. right rear variable function buttons

- CHGOBJ ⤴: rotates the objective turret one position clockwise in the current mode (dry or immersion)
- CHGOBJ ⤵: same as above but counterclockwise
- DRY/IMM: toggles between dry and immersion mode

## 5. right front: Focus buttons

- Z ↑: moves the Z drive up when a upper focus stop has been set
- Z ↓: moves the Z drive down
- SET + Z ↑: toggles set and unset upper focus stop
- SET + Z ↓: toggles set and unset lower focus stop





- SmartMove remote control for stage movements, as observed in the viewer display window of the software











1. X
2. Y
3. Z (focus, z-wide)
4. left button set selects Precise or Fast XY movement
5. right button set selects FINE or COARSE focus control




- front LCD panel displays the current status
  1. Contrasting method e.g., TL, FLUO, or SCAN; and IL SHUTTER status
  2. Objective lens and Magnification, see front top buttons above
  3. Illumination and Apertures
  4. Camera Ports/Eyepieces, see front top buttons above
  5. Focus control

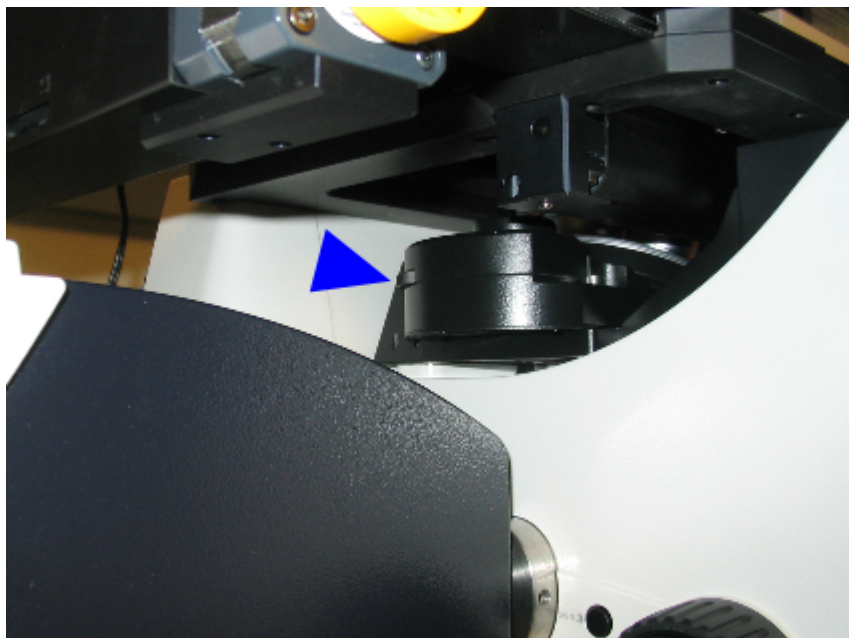
*Note:*

- display on the right shows z at 0  $\mu\text{m}$  with upper focus stop set and lower focus stop unset
- if upper focus stop is unset, display will indicate --- mm and 
-  indicates lower focus stop is set

|   |  |  |  |
|---|--|--|--|
| 1 |  | TL_BF  |   |
| 2 |  | 20 x Obj. IMM<br>1 x MagCh.  | $\Sigma$ 200 x   |
| 3 |  | INT 3<br>AP 13  |  |
| 4 |  |  100%           |  |
| 5 |  | 0 $\mu\text{m}$  |   coarse |






## Modes of operation

- TL
  1. press CHG TL  to rotate through BF, DIC, and POL
  2. adjust intensity (INT) and aperture (AP) as needed
  3. optional, press CHG CS to scan mode thus shutoff transmitted light
  4. a knurled wheel (arrowhead) on the left side below the objective turret adjusts the DIC shear; it's a rather tight space!



- IL/epifluorescence
  1. turn on FLUO (fluorescent light source)
  2. press the button for the desirable cube, see the front buttons in Controls
  3. press Shutter to illuminate specimen
  4. adjust the intensity (INT) and field diaphragm (FD) as needed
  5. press Shutter to block illumination when done viewing

## Using immersion objective lenses

- 2 ways to switch from DRY to IMM objectives
  - semi-auto with Mark and Find (recommended)
    1. locate the area of interest with a dry objective and focus
    2. click  to bring up the Mark and Find interface
    3. click  to record the current position: positionN
    4. press and hold Z ↓ to lower the turret completely
    5. switch immersion objective into light path
      - via the objective link in LAS AF (recommended)
      - or at the microscope: press DRY/IMM to switch mode, press either button CHGOBJ  or  to select the desired objective
    6. move the stage to expose the objective so you can apply immersion fluid
    7. apply an appropriate amount of immersion fluid
    8. select positionN from the drop down list and the stage should move back and bring the objective to focus
    9. click  to dismiss the Mark and Find interface
  - manual (not recommended)
    1. locate the area of interest with a dry objective and focus
    2. make sure upper focus stop is set
    3. press and hold Z ↓ to lower the turret completely
    4. switch immersion objective into light path (see semi-auto procedures above)
    5. offset the turret ~15 ° counterclockwise manually and apply a small drop of immersion fluid on the objective
    6. swing the turret back into position
    7. press and hold Z ↑ to put the prep back to the previously set focal plane
- when finish
  1. press and hold Z ↓ to lower the turret completely
  2. remove slide
  3. at the end of the session, use the provided lens paper to remove the excess immersion fluid from the front lens
  4. switch in the 10× objective
- Best practice
  - always set the upper focus stop whenever you put on a prep, press SET + Z ↑
  - switch objective lenses via LAS AF software or the right rear buttons on the microscope
  - if you want to manually switch the objective, DO NOT grab the objective lens barrel, use the turret instead

*Caution:* All our immersion objective lenses have aperture or correction collar, grabbing the barrel can easily move the collar and also strain the lenses.

  - select the 10× objective at the end of the session



more on immersion objective lenses

- objective with correction collar (the upper knurled ring with a black dot)



20×/0.7 NA multi immersion objective, currently set to use oil only

- OIL: oil, with or without coverglass
- GLYC: glycerol, with or without coverglass
- 0.17: water, with #1.5 coverglass
- W: ca. 3% NaCl, without coverglass
- 0: water, without coverglass



63×/1.2 NA water immersion objective, set at 0.17

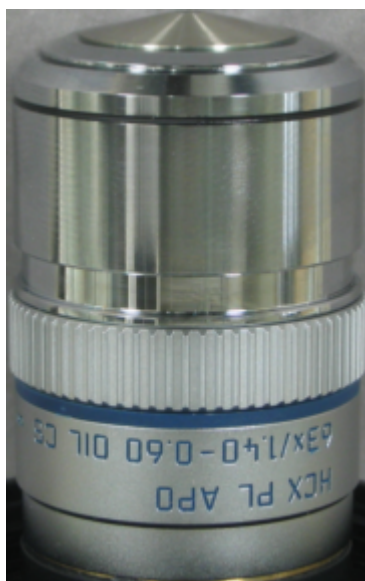
- T+ ←: turn to this direction for higher temperature or thinner coverglass
- 0.14 0.16 0.18: water, with specified coverglass thickness in mm
- → SW: turn to this direction for higher salt concentration, lower temperature, or thicker coverglass

*Note:* Please consult facility staff if it is the first time you want to adjust the correction collar, the poor design of this area of the inverted stand makes it almost impossible to adjust the collar.

- objective with iris diaphragm, controlled by turning the aperture collar (knurled ring)
  - these lens should be used with the iris diaphragm fully open for the highest NA
  - to fully open the iris turn the collar clockwise slowly until it clicks
  - the collar should have a little bit of resistance to turning at this fully open position



40×/1.25 NA oil immersion objective

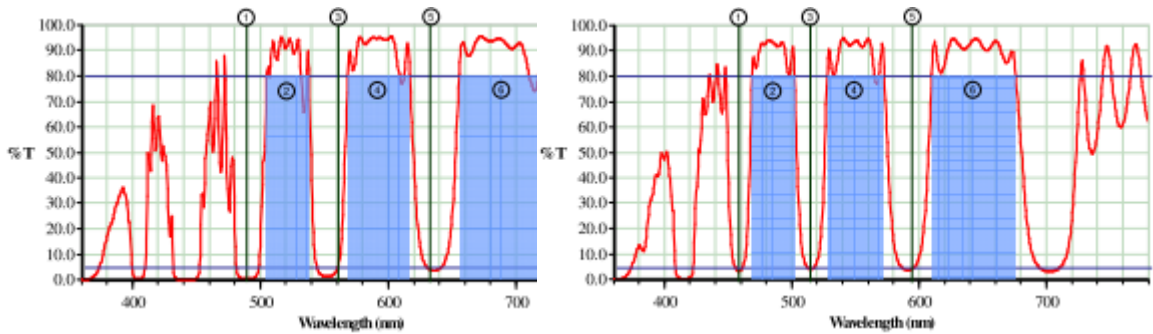


63×/1.4 NA oil immersion objective

Smart Panel (coming soon)

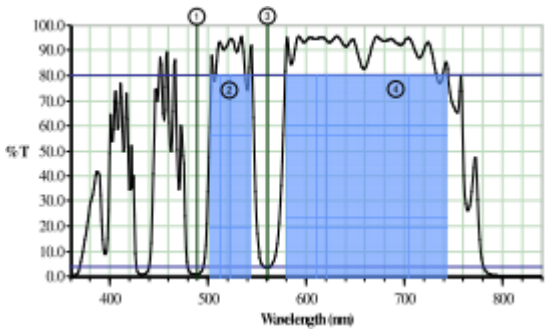
beam splitter spectra

- RT 30/70 is 30% reflection and 70% transmission
- Substrate is just plain glass (50/50)
- RSP 500 is a 500 nm long pass filter
- TD and DD are triple and double dichroics (transmission bands in blue)



TD 488/561/633

DD 458/514 is similar to the TD 458/514/594 shown here



DD 488/561

working with lif file

- use ImageJ and Bio-Formats plugins, see details on [using ImageJ](#)
- use the free [Leica LAX X software](#) for 64-bit Windows

scanner

dwell time at several common scan speed settings

| line frequency (Hz) | horizontal scan duration (s) | dwell time (μs) |      |
|---------------------|------------------------------|-----------------|------|
|                     |                              | max             | min  |
| 10                  | 0.1                          | 84.9            | 49.7 |
| 100                 | 0.01                         | 8.5             | 5.0  |
| 400                 | 0.025                        | 2.1             | 1.2  |
| 600                 | 0.0017                       | 1.4             | 0.8  |
| 1000                | 0.001                        | 0.9             | 0.5  |

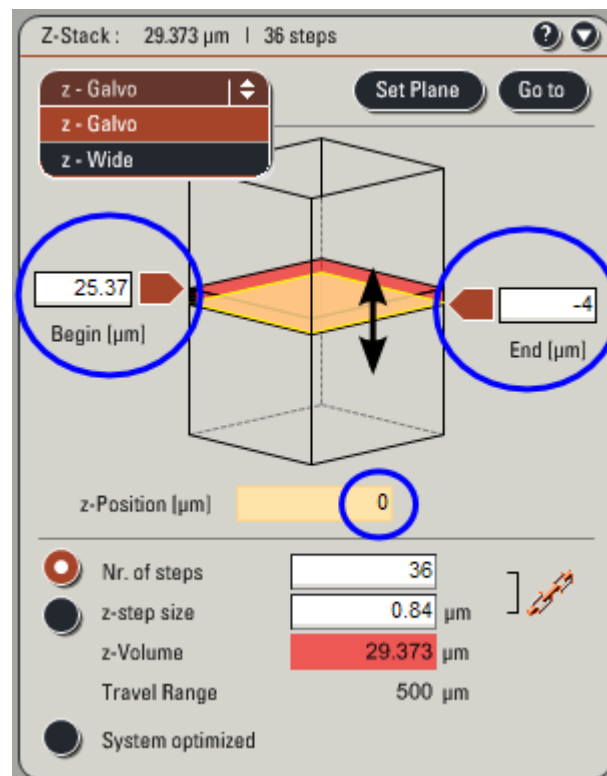
## Z

## software control of the z-drive

This provides alternate means to control the z-Position only when the Smart Panel is unavailable

- enter a number directly into the text boxes of z-Position, Begin, or End
- mouse: place cursor on z-plane (cursor change into double arrow), either click and drag, or click and then use thumb wheel

*Caution:* Use extreme caution with mouse control! It is very easy to drag the z-position too far and ram the objective onto the slide and damage the objective.



## setting z-step size or slice thickness

- the general guideline is to set the z-step size to  $\frac{1}{2}$  to  $\frac{1}{3}$  of the section thickness (clickable link) shown in the XY panel
  - section thickness (dz) is dependent on the emission wavelength ( $\lambda$ ), refractive index of the immersion medium (n) and NA of the objective, and the size of the pinhole (AU)
 
$$dz = \sqrt{(\lambda \times n \div NA^2)^2 + (AU \times n \times \sqrt{2} \times 1.22 \times \lambda \div NA^2)^2}$$
  - section thickness can be adjusted to the mean of the emission wavelength by following the link and then Apply to the step size
  - usually the shortest wavelength emission band is used to set the section thickness in a multichannel scan
- use System optimized in the Z-Stack panel if you plan to do deconvolution

